ISOLATION AND BIOCHEMICAL CHARACTERIZATION OF PROTEIN PROTEINASE INHIBITORS FROM *Cajanus cajan AND Phaseolus aureus*

Thesis
Submitted for the Award of the degree of

Doctor of Philosophy
in
Biotechnology

By

Soghra Khatun Haq

Under the Supervision of

Dr. Rizwan Hasan Khan

INTERDISCIPLINARY BIOTECHNOLOGY UNIT
ALIGARH MUSLIM UNIVERSITY
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SYNOPSIS

The ubiquitous occurrence of proteinase inhibitors (PIs) is a well-established fact. They are present in multiple forms in numerous tissues of animals and plants as well as in microorganisms. Proteinase inhibitors comprise one of the most abundant classes of natural, defence-related proteins often present in seeds and induced in certain plant tissues by herbivory or wounding. Most storage organs such as seeds and tubers contain 1 to 10% of their total proteins as PIs, which inhibit different types of enzymes. The term 'protein' inhibitor of proteinases could in principle denote any protein that diminishes the enzymatic activity of a proteinase. The activity of PIs is due to their capacity to form stable complexes with target proteinases, blocking, altering or preventing access to the enzyme active site.

Protein proteinase inhibitors have been classified into several families based on extensive homology among its members, topological relationships between the disulfide bridges and the location of the reactive site. Plant serine proteinase inhibitors that obey the standard mechanism are grouped into Soybean (Kunitz), Bowman-Birk, potato I and II, and squash families. Several other inhibitor families, such as barley, ragi 1 and 2, and thaumatin, have also been suggested.

Proteinase inhibitors find widespread applications ranging from prevention of unwanted proteolysis and active-site titrants to anti-cancer, anti-fungal and anti-viral agents. Their principal role, as envisaged in plant defense has generated considerable attention towards proteinase inhibitors (PIs) and amylase inhibitors (AIs) as possible tools of natural and engineered resistance against pests and pathogens. But prior to the production of transgenic plants (protected against as many pests as possible), a better understanding of the interaction of enzymes with their inhibitors is of key importance. Hence, in vitro biochemical studies are an important prerequisite to efficient gene transfer solutions.

Cajanus cajan (L.) Millsp. is one of the oldest food crops and ranks fifth in importance among edible legumes of the world. It is important in human nutrition as a rich source of dietary protein. However, preharvest damage due to insect pests on developing seed and postharvest losses due to storage pests are severe.

A protein proteinase inhibitor (PI) has been purified from *Cajanus cajan* (L.) (pigeon pea) by acetic-acid precipitation, salt fractionation and ion-exchange chromatography on a DEAE-Cellulose column. The content of *C. cajan* proteinase inhibitor was found to be 15mg/20g dry weight of pulse. The molecular weight of the inhibitor as determined by SDS-PAGE under reducing conditions was found to be about 14,000. It showed inhibitory activity towards proteolytic enzymes belonging to the serine protease group, namely trypsin and α-chymotrypsin. The inhibitory activity was stable over a wide range of pH (6-10) and temperature (30°C-95°C). Estimation of sulfhydryl groups yielded one free cysteine and atleast two disulfide linkages. N–terminal sequence homology suggests it belongs to the Kunitz inhibitor family. Structural analysis by circular dichroism shows that the inhibitor possesses a largely unordered structure.

A detailed analysis of the thermostability of *C. cajan* proteinase inhibitor was made by studying conformational changes in the inhibitor at neutral and acidic pH using CD spectroscopy. Disulfide bonds are believed to contribute towards protein stability, hence the effect of dithiothreitol on the *C. cajan* proteinase inhibitor was also examined at neutral as well as acidic pH. The protein inhibitor showed a reversible two-state (N⇔D) transition at neutral pH with a T_m approximately equal to 63°C. The negative CD band intensities at 200 nm (far-UV) and ~ 280 nm (near-UV) decreased as a result of thermal stress. The effect was more pronounced at low pH and in the presence of dithiothreitol. Only partial reversibility was observed under acidic conditions. Significant changes in the near- as well as far- ultraviolet CD spectrum were observed in the presence of dithiothreitol suggestive of the importance of disulfide linkages in maintaining the structure of *C. cajan* proteinase inhibitor.

Phaseolus aureus Roxb. (mung bean) or *Vigna radiata* (L.) Wilczek, is another pulse grown widely for use as a human food (as dry beans or fresh sprouts) and is the principal crop from which edible bean sprouts are produced. They contain between 19-25 percent protein, 60 percent carbohydrate and 4 percent fibre. They are also rich in lysine and offer appreciable amounts of potassium, calcium, magnesium, iron and traces of thiamin, riboflavin and niacin. It is a commonly consumed pulse in India that is plagued by weevils during storage.

A novel protein proteinase/amylase inhibitor from the dietary leguminous pulse *Phaseolus aureus* Roxb. was purified by acetic-acid precipitation, salt fractionation, ion-exchange chromatography (DEAE-Cellulose) and affinity chromatography on Trypsin-Sepharose column. *P. aureus* inhibitor was observed to be a bi-functional inhibitor since it exhibits inhibitory activity towards proteolytic enzymes, namely trypsin and α-chymotrypsin as well as α-amylase. Physico-chemical studies revealed that it was a 13-kD, helix-rich protein containinig approximately eight tyrosines, one tryptophan and two disulfide linked cysteine residues. N–terminal sequence alignment showed no homology to other protein proteinase inhibitors reported from *Phaseolus* sp. thereby suggesting that *P. aureus* proteinase/amylase inhibitor is a novel inhibitor. Inhibitory activity measurements and circular dichroism (CD) conformational studies showed that the inhibitor was quite stable even at extremely high temperatures and was only slightly affected by pH changes. Treatments with trypsin for varying time periods did not alter its protease inhibitory activity but caused some reduction in its amylase inhibitory activity.

Detergents (cationic, anionic, zwitterionic and nonionic) have gained an important status in protein biochemistry and are applied in the biosciences and in many (bio) technological processes owing to the fact that detergents mimic the native, hydrophobic environment of the phospholipid bilayer *in vivo*. The effect of sodium dodecyl sulphate (anionic), sodium deoxycholate (anionic bile salt) and CHAPS (zwitterionic) detergents on the conformation of these proteinaceous inhibitors was investigated using circular dichroism spectroscopy. SDS causes minimal changes in the tertiary as well as secondary structure of *C. cajan* proteinase inhibitor. In the presence of sodium deoxycholate, minor changes in the far-UV CD spectrum were accompanied by loss in inhibitory activity while CHAPS did not affect the inhibitor function. As judged from the changes in circular dichroic curves (Θ_{MRW} at 208 and 222nm), the primarily disorganized polypeptide chain of *C. cajan* proteinase inhibitor was converted by HFIP into helical conformation. The *P. aureus* inhibitor showed increased helicity in the presence of SDS (Θ_{MRW} at 208 nm) as well as DOC and CHAPS (Θ_{MRW} at 222 nm). Fluorescence measurements show slight alterations in the emission intensities. HFIP caused a cooperative increase in α-helical secondary structure in the *P. aureus* inhibitor.

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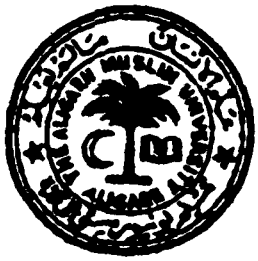

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Certificate

I certify that the work embodied in the thesis entitled "**Isolation and Biochemical Characterization of Protein Proteinase Inhibitors from *Cajanus cajan* and *Phaseolus aureus***" has been carried out by **Ms. Soghra Khatun Haq** under my supervision and that it is suitable for the award of Ph.D. degree in Biotechnology of the Aligarh Muslim University, Aligarh.

Rizwan Hasan Khan 18/11/09

Dr. Rizwan Hasan Khan
(Supervisor)

Declaration

I hereby declare that the thesis entitled "**Isolation and Biochemical Characterization of Protein Proteinase Inhibitors from *Cajanus cajan* and *Phaseolus aureus***", embodies the work carried out by me.

Soghra Khatun

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CONTENTS

3. Results & Discussion

Abstract

ABSTRACT

Protein proteinase inhibitors (PIs) comprise one of the most abundant classes of defense-related proteins in plants. Most storage organs such as seeds and tubers contain 1 to 10% of their total proteins as PIs, which inhibit different types of enzymes. *Cajanus cajan* (L.) Millsp. (pigeon pea) is one of the oldest food crops and ranks fifth in importance among edible legumes of the world. It is important in human nutrition as a rich source of dietary protein. *Phaseolus aureus* Roxb. (mung bean), also known as *Vigna radiata* (L.) Wilczek is also grown widely for use as a human food (as dry beans or fresh sprouts), as well as a green manure crop and as forage for livestock. However, preharvest damage due to insect pests on developing seed and postharvest losses due to storage pests are severe. Consequently, the amylase inhibitors (AIs) and proteinase inhibitors (PIs) have gained attention as possible tools of natural and engineered resistance against pests and pathogens.

A protein proteinase inhibitor (PI) has been purified from *Cajanus cajan* (L.) by acetic-acid precipitation, salt fractionation and ion-exchange chromatography on a DEAE-Cellulose column. The content of *C. cajan* proteinase inhibitor was found to be 15mg/20g dry weight of pulse. The molecular weight of the inhibitor as determined by SDS-PAGE under reducing conditions was found to be about 14,000. It showed inhibitory activity towards proteolytic enzymes belonging to the serine protease group, namely trypsin and α-chymotrypsin. The inhibitory activity was stable over a wide range of pH (6-10) and temperature (30°C-95°C). The inhibitor contains approximately two tryptophans and two tyrosines. Estimation of sulfhydryl groups yielded one free cysteine and atleast two disulfide linkages. N–terminal sequence homology suggests it belongs to the Kunitz inhibitor family. Structural analysis by circular dichroism revealed the absence of any regular α-helical or β-sheet structure. Instead the inhibitor was seen to possess a largely unordered structure.

A detailed analysis of the thermostability of *C. cajan* proteinase inhibitor was made by studying conformational changes in the inhibitor at neutral and acidic pH using CD spectroscopy. Disulfide bonds are believed to contribute towards protein stability, hence the effect of dithiothreitol on the *C. cajan* proteinase inhibitor was also examined

at neutral as well as acidic pH. The protein inhibitor showed a reversible two-state (N↔D) transition at neutral pH with a T_m approximately equal to 63°C. The negative CD band intensities at 200 nm (far-UV) and ~ 280 nm (near-UV) decreased as a result of thermal stress. The effect was more pronounced at low pH and in the presence of dithiothreitol. Only partial reversibility was observed under acidic conditions. Significant changes in the near- as well as far- ultraviolet CD spectrum were observed in the presence of dithiothreitol suggestive of the importance of disulfide linkages in maintaining the structure of *C.cajan* proteinase inhibitor.

A novel protein proteinase/amylase inhibitor from the dietary leguminous pulse *Phaseolus aureus* Roxb. (mung bean), also known as *Vigna radiata* (L.) Wilczek was purified by acetic-acid precipitation, salt fractionation, ion-exchange chromatography (DEAE-Cellulose) and affinity chromatography on Trypsin-Sepharose column. Test of inhibitory activity towards enzymes of different mechanistic classes revealed that *P. aureus* inhibitor is a bi-functional inhibitor since it exhibits inhibitory activity towards (serine) proteolytic enzymes, namely trypsin and α-chymotrypsin as well as an amylolytic enzyme, namely α-amylase. It is a 13-kD protein containing approximately eight tyrosines, one tryptophan and one free and two disulfide linked cysteine residues. Conformational studies using circular dichroism measurements revealed that *P. aureus* proteinase/amylase inhibitor possessed α-helical secondary structure. Inhibitory activity measurements and CD (circular dichroism) conformational studies show that the inhibitor is quite stable even at extremely high temperatures and is only slightly affected by pH changes. Treatments with trypsin for varying time periods did not alter its protease inhibitory activity but caused some reduction in its amylase inhibitory activity. N–terminal sequence alignment reveals no homology to other protein proteinase inhibitors reported from *Phaseolus* sp. thereby suggesting that it is a novel inhibitor.

Detergents (cationic, anionic, zwitterionic and nonionic) have gained an important status in protein biochemistry and are applied in the biosciences and in many (bio) technological processes owing to the fact that detergents mimic the native, hydrophobic environment of the phospholipid bilayer *in vivo*. Keeping in view the wide applicability of detergents in membrane protein solubilization, electrophoresis, etc., it becomes imperative to develop a better understanding of the effects of detergents on proteins

Moreover, proteinase inhibitors and detergents are routinely used together in cell lysis buffers to inhibit unwanted proteolysis and facilitate membrane protein solubilization in protein purification procedures. It is therefore essential to recognize the detergents that will accomplish the task of maintaining solubility while not significantly compromising the native structure and consequently the biological function of the protein.

The effect of sodium dodecyl sulphate (anionic), sodium deoxycholate (anionic bile salt) and CHAPS (zwitterionic) detergents on the conformation of these proteinaceous inhibitors was investigated using circular dichroism spectroscopy. SDS causes minimal changes in the tertiary as well as secondary structure of *C. cajan* proteinase inhibitor. In the presence of anionic bile salt, deoxycholate, minor changes in the far-UV CD spectrum were accompanied by loss in inhibitory activity while CHAPS did not affect the inhibitor function. As judged from the changes in circular dichroism curves ($[\Theta]_{MRW}$ at 208 and 222nm), the primarily disorganized polypeptide chain of *C. cajan* proteinase inhibitor was converted by HFIP into helical conformation.

The *P. aureus* inhibitor showed increased helicity in the presence of SDS ($[\Theta]_{MRW}$ at 208nm) as well as DOC and CHAPS ($[\Theta]_{MRW}$ at 222nm). Fluorescence measurements show slight alterations in the emission intensities. HFIP caused a cooperative increase in α-helical secondary structure in the *P. aureus* inhibitor.

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I owe the financial assistance for carrying out the work embodied in this thesis to the **Council of Scientific and Industrial Research (CSIR), New Delhi.**

Commission and omissions are mine.

Soghra Khatun

Soghra Khatun Haq


All that I am or hope to be,
I owe to my mother....
&
to my father....
(for always making me feel so special)

It is Allah Who causes the seed grain and the date- stone to split and sprout. He causes the living to issue from the dead and He is the One to cause the dead to issue from the living. That is Allah: then how are you eluded away from the Truth? (Al-An'aam:95)

It is He Who sends down rain from the skies: with it We produce vegetation of all kinds: from some We produce green (crops), out of which We produce grain, heaped up (at harvest); out of the date-palm and its sheaths (or spathes) (Come) clusters of dates hanging low and near; And (then there are) gardens of grapes and olives, and pomegranates, each similar (in kind) yet different (in variety): when they begin to bear fruit, feast your eyes with the fruit and the ripeness thereof. Behold! In these things there are signs for people who believe.
(Al-An'aam:99)

Does man not see that it is We who created him from sperm? Yet, behold! He stands forth as an open adversary! And he makes comparisons for Us, and forgets his own origin and Creation. He says, "Who can give life to dry bones and decomposed ones (at that)? Say, "He will give them life Who created them for the first time! For He is Well-versed in every kind of creation." (Ya-seen: 77-79)

"We created man from a quintessence (of clay); Then We placed him as (a drop of) sperm in a place of rest, firmly fixed; Then We made the sperm into a congealed blood; then that clot We made a (fetus) lump; then We made out of that lump bones and clothed the bones with flesh; then We developed out of it another creature. So blessed be Allah, the noblest of Creators!" (Al-Mumenoon: 12-14)

"Does man think that he will be left uncontrolled, without purpose? Was he not a drop of sperm emitted in lowly form? Then he became a leech-like clot; then Allah made and fashioned him in due proportion, And of him He made two sexes, male and female. Has not He, the same, the power to give life to the dead?" (Al-Qiyama: 36-40)

It is We Who have created you: why then will you not witness the Truth? Do you then see?---- The human seed that you discharge--- Is it you who create it, or We? We have decreed Death to be your common lot, and We are not to be frustrated……And you certainly know already the first form of creation: why then ye not celebrate His praises? Consider the seed that you sow in the ground. Is it you that cause it to grow, or are We the Cause? Were it Our Will, We could crumble it to dry powder, and you would be left in wonderment…..Consider the water which you drink. Do you bring it down as rain from the cloud, or do We? Were it Our Will, We could make it salt and unpalatable: then why do you not give thanks? Observe the fire which you kindle. Is it you who grow the tree which feeds the fire, or do We grow it?….
….Then celebrate with praises the name of your Lord, the Supreme! (Al-Waqia: 57-75)

…….The Glorious Qur an
!!!

Abbreviations

ABA	: Abscissic acid
AI	:Amylase inhibitor
AIDS	:Acquired immuno-deficiency syndrome
BAPNA	:N_{α}-Benzoyl-L-Arg-p-nitroanilide
Bt	:Bacillus thuringiensis
CaMV	:Cauliflower mosaic virus
CD	:Circular dichroism
CHAPS	:3-[(3-cholamidopropy) dimethylammonio]-1-propane sulfonate
CpTI	:Cowpea trypsin inhibitor
3,4-DCI	:3,4-Dichloroisocoumarin
DEAE	:Diethyl amino ethyl
DFP	:Disopropylfluorophosphate
DNS	:Dinitrosalicylate
DOC	:Deoxycholate
Cry	:Crystal
DTT	:Dithiothreitol
DTNB	:5,5' Dithiobis nitrobenzoic acid
HFIP	:3,3,3,3',3',3'-hexafluoro-2-propanol
HIV	:Human immunodeficiency virus
HSV	:Herpes simplex virus
IPM	:Integrated pest management
JA	:Jasmonic acid
MAPK	:Mitogen activated protein kinase
MTI	:Mustard trypsin inhibitor
NATA	:N-Acetyl-L-tryptophanamide
OC	:Oryzacystatin
PCD	:Programmed cell death
PMSF	:Phenylmethylsulfonylfluoride
PI	:Proteinase inhibitor
PIIF	:Proteinase inhibitor initiation factor
PTH	:Phenylthiohydantoin
PVY	:Potato virus Y
SDS-PAGE	:Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SKTI	:Soybean kunitz trypsin inhibitor
STI	:Soybean trypsin inhibitor
TCA	:Trichloroacetic acid
TEV	:Tobacco etch virus
TPCK	:Tosyl $_{L}$-Phe chloromethylketone
UV-Vis	:Ultraviolet-Visible
VIP	:Vegetative insecticidal proteins

LIST OF FIGURES

PageNo.

PageNo.

LIST OF TABLES

Introduction

1. INTRODUCTION

The ubiquitous occurrence of proteinase inhibitors (PIs) is a well-established fact. They are present in multiple forms in numerous tissues of animals and plants as well as in microorganisms. Proteinase inhibitors comprise one of the most abundant classes of natural, defence-related proteins often present in seeds and induced in certain plant tissues by herbivory or wounding [1-3]. Most storage organs such as seeds and tubers contain 1 to 10% of their total proteins as PIs, which inhibit different types of enzymes [4,5]. The term 'protein' inhibitor of proteinases could in principle denote any protein that diminishes the enzymatic activity of a proteinase. The activity of PIs is due to their capacity to form stable complexes with target proteinases, blocking, altering or preventing access to the enzyme active site.

1.1. PLANT PROTEIN PROTEINASE INHIBITORS (MECHANISTIC CLASSIFICATION)

The inhibitors discovered so far have been found to be specific for each of the four mechanistic classes of proteolytic enzymes, and based on the active amino acid in their "reaction center" [6] are classified as serine, cysteine, aspartic and metallo-protease inhibitors.

Serine proteinase inhibitors

Serine proteinase inhibitors are universal throughout the plant kingdom and have been described in many plant species. Therefore the number of known and partially characterized inhibitors of serine proteinases is enormous. Serine proteinase inhibitors have been reported from a variety of plant sources [7-17] and are the most-studied class of proteinase inhibitors. Serine proteinase inhibitors are widespread in the plant kingdom, their physiological roles including the regulation of endogenous proteinases during seed dormancy, the reserve protein mobilization, and the protection against the proteolytic enzymes of parasites and insects. Moreover, they may also act as storage or reserve proteins.

The two best characterized families of plant serine proteinase inhibitors are the Kunitz-type and Bowman-Birk inhibitors. Kunitz-type inhibitors have a molecular mass of 18-22 kDa, one or two polypeptide chains, a low cystine content (usually with four Cys residues in two disulfide bridges) and one reactive site. In contrast, Bowman-Birk

type inhibitors have a lower molecular mass (8-10 kDa), a high cystine content and two reactive sites [18]. A common feature of plant PIs is high level of S-S (disulfide) bridges that confer great stability to heat, pH extremes, and to hydrolysis by proteases. The uniqueness of Bowman-Birk type inhibitors is that they have very little ordered structure in them (α-helix and β- structure) but have high content of aperiodic structure which offers greater flexibility to the molecule. Crystal structure analysis of soybean trypsin inhibitor (Kunitz) has also revealed the absence of α- helix and most of the polypeptide chain is involved in approximate β-pleated sheet structures with little, if any, regular sheet structure [19].

Cysteine proteinase inhibitors

Plant cystatins or phytocystatins are the second most-studied class of inhibitors and have been identified and characterized from several plants, *viz.*, cowpea [20], potato [21], cabbage [22], ragweed [23], carrot [24], papaya [25], apple fruit [26], avocado [27], chestnut [28] and Job's tears [29] etc. Cystatins have also been isolated from seeds of a wide range of crop plants. These crop plants include those of sunflower [30], rice [31], wheat [32], maize [33], soybean [34], sugarcane [35] etc.

Aspartic proteinase inhibitors

Aspartic proteinase inhibitors are a relatively less-studied class partly due to their rarity of occurence. Potato tubers possess an aspartic proteinase (cathepsin D) inhibitor, [36] that shares considerable amino acid sequence identity with the trypsin inhibitor SBTI from soybeans. It is the only well-characterized cathepsin D inhibitor of protein character.

Metallo-proteinase inhibitors

The metallo-proteinase inhibitors in plants are represented by the metallo-carboxypeptidase inhibitor family in tomato [37] and potato plants [38,39].

1.2. THE INHIBITOR FAMILIES

Laskowski and Kato have classified the proteinase inhibitors into several families based on extensive homology among its members, topological relationships between the disulfide bridges and the location of the reactive site. Plant serine proteinase inhibitors that obey the standard mechanism (as discussed later) are grouped into Soybean (Kunitz),

TABLE-I

The Inhibitor Families of Plant Origin[a]

	Name	Reference
I.	Soybean trypsin inhibitor (Kunitz) family	[43]
II.	Soybean trypsin inhibitor (Bowman-Birk) family	[6,44]
III.	Potato I inhibitor family	[45]
IV.	Potato II inhibitor family	[46]
V.	Squash family	[47]
VI.	Other families	
	Barley family	
	Cereal superfamily	
	Thaumatin-like inhibitors	
	Ragi A1 inhibitors	

[a] Adapted from Laskowski & Kato [40] with slight modification

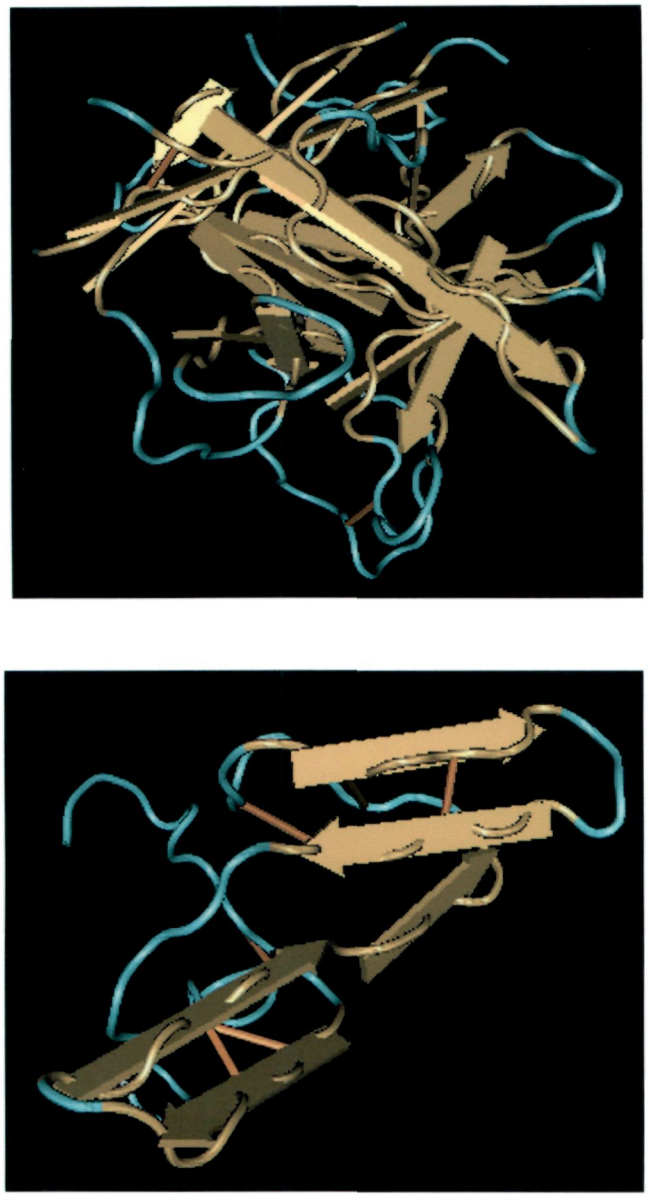


Fig.1 Three-dimensional view of a Kunitz inhibitor (upper panel) and a Bowman-Birk inhibitor (lower panel) showing secondary structural elements and connecting disulfide bridges [48,49].

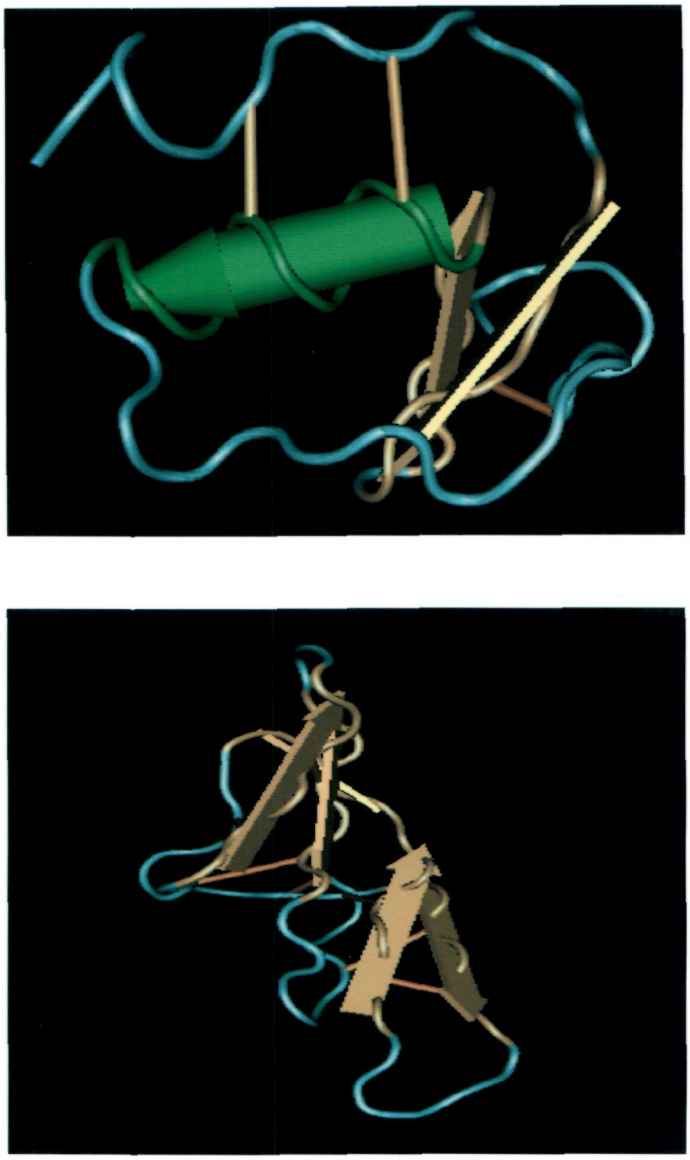


Fig.2 Three-dimensional view of a Kazal inhibitor (upper panel) and a bifunctional soybean Bowman-Birk inhibitor (lower panel) showing secondary structural elements and connecting disulfide bridges [50,51].

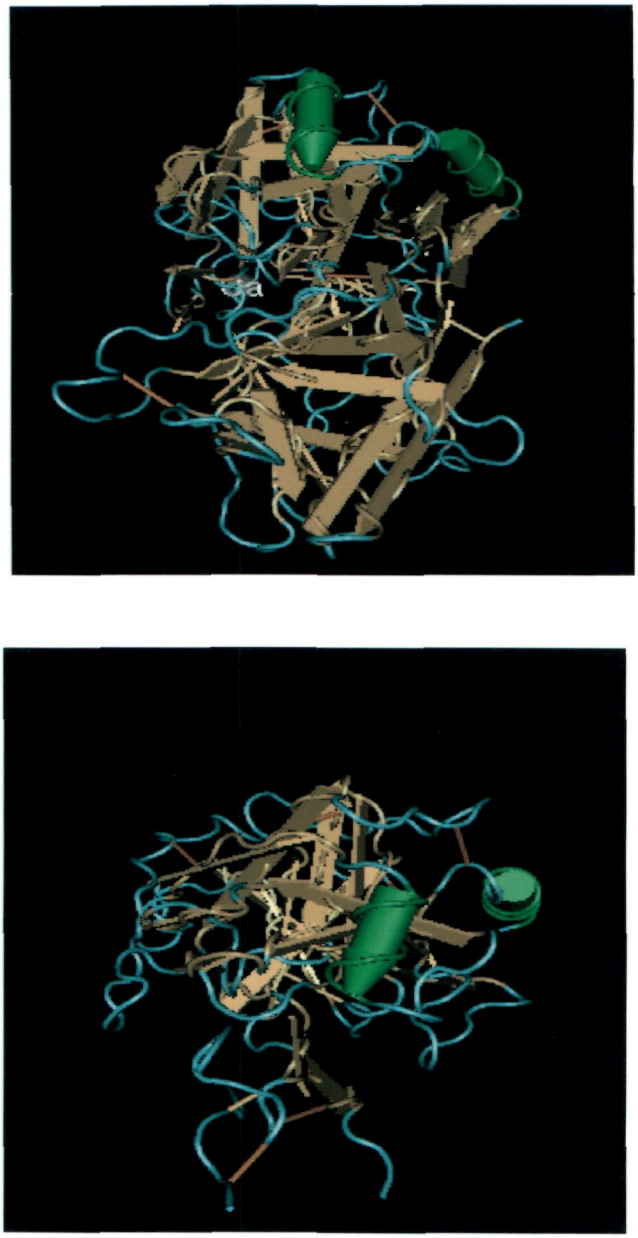


Fig.3 Three-dimensional view of a Kunitz inhibitor (upper panel) and a Bowman-Birk inhibitor (lower panel) complexed with trypsin [52,53].

Bowman-Birk, potato I and II, and squash families [40]. Several other inhibitor families, such as barley, ragi 1 and 2, and thaumatin, have also been suggested [41,42].

1.3. THE STANDARD MECHANISM

Extensive research on proteinase inhibitors has provided a basic understanding of the mechanism of action that applies to most serine proteinase inhibitor families and probably to the cysteine and aspartyl proteinase inhibitor families as well. All serine inhibitor families from plants are competitive inhibitors and all of them inhibit proteinases with a similar standard mechanism [40] as proposed by Laskowski and Kato. Inhibition occurs as a consequence of binding of the active-site substrate-binding region of a proteinase to the corresponding substrate-like region (reactive site) on the surface of the inhibitor [54]. At or near the center of the reactive site is an amino-acid residue specifically recognized by the primary substrate binding site of the target proteinase and termed the P_1 residue [55]. Adjacent to P_1 in the direction of the carboxy terminus is the residue referred to as P_1'. It is the peptide bond joining these two residues, known as the reactive-site peptide bond, which is hydrolyzed during complex formation between an inhibitor and its target enzyme. The overall mechanism of the enzyme-inhibitor interaction, including only those intermediates whose existence was definitely shown can be written as:

$$E + I \Leftrightarrow L \Leftrightarrow C \Leftrightarrow X \Leftrightarrow L^* \Leftrightarrow E + I^*$$

where E is the enzyme, I and I* virgin (reactive site peptide bond intact) and modified (reactive site peptide bond hydrolyzed) inhibitors, respectively. L and L* are loose, noncovalent (rapidly dissociable) complexes [56] of E with I and I*, respectively, X is the relatively long-lived intermediate in the E+I* reaction [57], and C is the stable enzyme-inhibitor complex.

Therefore, inhibitors behave as highly specific, limited proteolysis substrates for their target enzymes. On the surface of each inhibitor molecule lies at least one (more in multiheaded inhibitors) peptide bond called the reactive site [54], which specifically interacts with the active site of the cognate enzyme. The value of k_{cat}/K_m for the hydrolysis of this peptide bond by the cognate enzyme at neutral pH is very high, 10^4 - 10^6 $M^{-1}s^{-1}$ [58], compared to a typical value for normal substrates of about 10^3 $M^{-1}s^{-1}$. However for inhibitors, the values of k_{cat} and K_m are both many orders of magnitude

lower than the values for normal substrates. Therefore their hydrolysis is extremely slow, and the system behaves as if it were a simple equilibrium between the enzyme and free inhibitor on the one hand and the complex on the other.

On the basis of elegant studies on various crystal structures of enzyme-inhibitor complexes [59], Bode and Huber showed that inhibitors bind to their cognate enzymes in either a "substrate canonical form" or a "product canonical form".

1.4. THE NATURE OF THE REACTIVE SITE

X-ray crystallographic studies of pancreatic trypsin inhibitor (Kunitz) [60], soybean trypsin inhibitor (Kunitz) [19], and subtilisin inhibitor, S-SI [61], permit comparison of the three-dimensional structure of these inhibitors. Although the sequences of all the three inhibitors are quite different, the geometry of the region surrounding the reactive site [62] is virtually identical. The amino-acid sequences surrounding the reactive sites of the various families are summarized in Table-II. In all inhibitors that clearly obey the standard mechanism, the reactive site peptide bond is encompassed in at least one disulfide loop [53,63], which ensures that during conversion of virgin to modified inhibitor the two peptide chains cannot dissociate. However, this requirement is not absolute since soybean Kunitz inhibitor (Kunitz) with its Met^{84}-Leu peptide bond hydrolyzed by subtilisin is still fully inhibitory, and can be converted to modified inhibitor (Arg^{63}-Ile reactive site hydrolyzed) without loss of the 64-84 fragment [64].

The reactive site peptide bond after hydrolysis (i.e. in the modified inhibitor) acquires a newly formed carboxyl terminal residue designated as P_1 and a newly formed amino terminal residue designated as P_1'. The reactive site residue P_1, generally corresponds to the specificity of the cognate enzyme. Thus inhibitors with P_1 Lys and Arg tend to inhibit trypsin and trypsin-like enzymes, those with P_1 Tyr, Phe, Trp (artificial only), Leu and Met inhibit chymotrypsin and chymotrypsin-like enzymes, and those with P_1 Ala and Ser inhibit elastase-like enzymes. In the Kazal secretory inhibitor family, inhibitors with P_1 Leu and Met are strong inhibitors of elastase [65]; in the Bowman-Birk family elastase is inhibited with P_1 Ala, but not with P_1 Leu [66,67]. In many instances strong inhibitors of trypsin with P_1 Arg and Lys inhibit chymotrypsin on the same reactive site [66,68,69]. The ability to tolerate a synthetic or mutational replacement of the P_1 residue and still

TABLE-II

Alternative amino acid residues in the sequence surrounding the reactive site (arrow) of inhibitors*

	P_4	P_3	P_2	P_1 ↓	P_1'	P_2'	P_3'	P_4'
Pancreatic Trypsin Inhibitor (Kunitz) family	...G	P	C	K	A	R	I	I...
		L		R	G	A	L	P
		R		Y	R	S	F	L
		N		M	Q	Y	T	R
Pancreatic Trypsin Inhibitor (Kazal) Family	...G	C	P	R	I	Y	N	P...
	V		N	K	D	L	R	L
	A		T	E	E	P	H	F
	L		A	D	A	F	Q	R
	D		M	A	L	H	S	E
	M		L	L	Q	D	M	
	F		S	S	N	Q	K	
***Streptomyces* Subtilisin Inhibitor Family**	... M	C	P	M	V	Y	D	P...
	A		T	K	Q	F		
Bowman-Birk Inhibitor Family	... A	C	T	K	S	N	P	P...
	M		A	R		M		G
	V			A		I		A
	L			L	Q			
	I			F	Y			
Soybean Trypsin Inhibitor (Kunitz) Family	... P	S	Y	R	I	R	F	I...
Potato Inhibitor I Family	... P	V	T	L	D	Y	R	C...
Potato Inhibitor II Family	... A	S	Y	K	S	V	C	E...
Alpha-1-Proteinase Inhibitor Family	... A	I	P	M	T	I	P	P...

*Adapted from Laskowski & Kato [40]

retain inhibitory activity, either with retention of original specificity (e.g. after Arg → Lys or Leu → Met exchanges) or with dramatic change in specificity (eg. tryptic to chymotryptic after Arg ↔Trp exchanges), is peculiar to proteinase inhibitors.

1.5. GEOMETRY OF THE REACTIVE SITE

The detailed molecular events at the active site of the enzyme and at the reactive site of the inhibitor as elucidated by Huber and his group [70] shows that in the stable enzyme-inhibitor complex, the reactive site scissile peptide bond (P_1-P_1') of the inhibitor is intact, but the carbonyl carbon is no longer trigonal. It is appreciably pyramidalized by the interaction of its oxygen with the "oxyanion hole" of the enzyme [71]. The conformation of residues surrounding the reactive site peptide bond is that of an optimal substrate. Reactive sites are very rigid. Upon enzyme-inhibitor interaction, conformational changes in either partner is minimal; it is almost a lock and key interaction. Besides this, other interactions such as H-bonds, van der Waals forces and possibly salt bridges contribute to the characteristic stability of the complex.

1.6. MULTIPLE REACTIVE SITES ON A SINGLE POLYPEPTIDE CHAIN

When two (or more) enzymes can be inhibited by the same inhibitor molecule, two operationally distinguishable situations can arise. The enzymes can compete for the inhibitor or they can be inhibited simultaneously and independently. In the competitive case, we can envisage either inhibition on the same reactive site or inhibition on two distinct, overlapping reactive sites where steric hindrance prevents association with both enzymes. Overlapping reactive sites seems to be a plausible explanation, however there is no convincing example. Numerous cases of independent inhibition of several enzymes are known. Functionally, inhibitors are classified as single-headed if they have only one active reactive site, double-headed if they have two and so on [72]. The most common method of achieving multiheadedness is by gene duplication, however elongation by gene fusion may also be possible. The first such case discovered was lima bean Bowman-Birk inhibitor [73].

1.7. INDUCTION OF PI SYNTHESIS AND ITS REGULATION IN PLANTS

Plants produce protease inhibitors in response to feeding or wounding that are usually active against the endoproteases [5]. The protease inhibitors are also developmentally regulated as has been observed in case of cabbage and sweet potato [74-

76]. Highest levels are found in very young leaves while older leaves have the lowest levels, also higher in the cabbage head.

Earlier research indicated that wound-induced accumulation of proteinase inhibitors in tomato was mediated by a proteinase inhibitor initiation factor (PIIF) that switched on a cascade of events leading to the synthesis of the inhibitor proteins [77,78] that were primarily cytosolic [79,80]. As reported by Lawrence and Koundal [81], in their review, current evidence suggests that the production of the inhibitors occurs via the octadecanoid (OD) pathway, which catalyzes the breakdown of linolenic acid and the formation of jasmonic acid (JA) to induce proteinase inhibitor gene expression. There are four systemic signals responsible for the translocation of the wound response,(through the xylem or phloem), which includes systemin, abscissic acid (ABA), hydraulic signals (variation potentials) and electrical signals [82-84]. Evidence of systemin playing a key role in systemic signaling came from the pioneering work of C.A. Ryan. By showing that tomato plants expressing an antisense prosystemin gene become deficient in long-distance signaling and are more susceptible to insect attacks than wild-type plants [85]; an important role of systemin in defence signalling pathways was clearly established. Immunolocalization techniques revealed that prosystemin, the precursor of systemin found in several plants [86], is localized in parenchyma cells of vascular bundles [87], this localization in the vicinity of the sieve tubes of the phloem may facilitate transport of systemin and oxylipins it induces in response to wounding to distal cells. The activation of defensive genes by systemins involves a complex intracellular signaling pathway akin to the inflammatory response in animals. Systemin interacts with a 160-kDa cell surface receptor (SR160) that leads to the activation of a mitogen-activated protein kinase (MAPK) [88-90], the rapid alkalinization of the extracellular medium [91], the activation of phospholipase [92,93] and the release of linolenic acid that is converted into oxylipins such as phytodienoic acid and jasmonic acid that are powerful signals for defense genes. [94-95]. Transgenic potato plants overexpressing the prosystemin gene were found to regulate the synthesis and accumulation of proteinase inhibitors in leaves [96]. Paralogous to the induction and synthesis of tomato inhibitor-II in tomato leaves in response to wounding; tobacco leaves synthesize a tobacco trypsin inhibitor (TTI) in response to wounding suggesting a similarity between the wound signaling systems of the

two plants. The search for a systemin-like signaling molecule by Ryan and Pearce [97] yielded tobacco hydroxyproline-rich proteins (TobHypSys) that were identified as members of a functionally related systemin family.

1.8. PIs CONFER RESISTANCE TOWARDS INSECT INFESTATION

Subsequent to the preliminary observation of Mickel and Standish of the role of soybean products on crop protection; the trypsin inhibitors present in soybean were shown to be toxic to the larvae of flour beetle, *Tribolium confusum* [98]. Following these early studies, there have been many examples of protease inhibitors active against certain insect species, both *in vitro* assays against insect gut proteases [12,99] and *in vivo* artificial diet bioassays [100-102]. Initially, one PI member of the plant cysteine protease inhibitors, oryzacystatin (gene OC I), showed a low but significant growth inhibition activity on many aphid species tested *in vitro*. Furthermore, transgenic oilseed rape plants expressing OC I in their phloem sap affected similarly the larval growth of *Myzus persicae*, and induced significant reductions in aphid fecundity [103,104]. Finally, tissue and enzyme targets of OC I were successfully identified by immunolocalization and enzymatic assays on OC I-fed aphids and cysteine-type proteases were shown to form the major endoproteolytic system in the aphid digestive tract. Also, a pea seed Bowman-Birk trypsin/chymotrypsin inhibitor was shown to induce significant *in vitro* toxicity towards the pea aphid *Acyrthosiphon pisum* [105].

1.9. MECHANISM OF ACTION IN INSECT GUTS

There are differing views on the mechanism of development of resistance. Reese [106] had proposed the simple mechanism that growth rates were reduced due to reduced rates of proteolysis that was later dismissed when Broadway and Duffey [107] suggested that a feedback mechanism was leading to the hyperproduction of proteinases to compensate for the loss of activity, which in turn led to the depletion of essential amino-acids and finally resulted in retarted growth rates. It was found that rats and chicks fed on low soya protein or STI (soybean trypsin inhibitor) extract developed hyperactive pancreas and their intestines contained greater amounts of pancreatic enzymes including trypsin even though a large percentage was inhibited. So depression of growth was not due to blocking of proteolysis, but to hyperactive pancreas. Nevertheless, the primary site of action of these inhibitors is the digestive system of insect larvae. Since trypsin is

involved in developmental processes such as molting and synthesis of neuropeptides, trypsin inhibitors can disrupt these processes thereby retarding growth and development of the larvae [106,108,109]. In a study conducted by Marchetti *et al.* [110], it was observed that larvae fed on transgenic plants expressing a Kunitz inhibitor, gradually lost their turgor and became shrunken; hence it appears that food avoidance also has a dramatic effect on the water balance of the feeding larvae.

1.10. NUTRITIONAL SIGNIFICANCE

Since inhibitor blocks intestinal protease, at first consideration one is lead to think that PI would cause malabsorption of amino acids and lack of growth in animals. However, studies on a range of experimental animals show that this is not the case. Although STI inhibits almost all trypsins *in vitro*, it does not cause poor growth in all animals. STI blocks trypsin leading to pancreas feedback mechanism; pancreas goes into hyper-production of pancreatic enzymes causing rapid depletion/loss of essential nutrients. However, it is only a serious problem in animals with a simple feedback/control of pancreas secretion. Humans and cattle have more sophisticated systems involving several other proteases; thereby eliminating doubts of growth limiting problems in mammals [111]. There is no evidence that PIs have toxic or deleterious effects on mammals. Rather, they are known to improve the nutritional quality [41]. Also, since it is well-known that legume proteins are deficient in the sulfur containing amino-acids [112], Biermann *et al.* [113] attempted to increase the sulfur content by adding Met or Sulfate that in turn led to increased mRNA levels of PIs; thus suggesting a positive role of PIs in nutrition. Many of these protease inhibitors are rich in cysteine and lysine, contributing to better and enhanced nutritional quality. Large amounts of inhibitors are normally present in many raw foods of plant origin, but their anti-nutritional effect is drastically reduced with simple technological treatments such as cooking. The oral administration of small quantities of certain inhibitors (e.g. soybean Bowman-Birk) has a protective role against carcinogenesis in the esophagus, duodenum and colon [114,115].

1.11. WHY PURIFY PROTEINASE INHIBITORS ?

Inhibition of unwanted proteolysis

All cells contain a variety of intracellular proteases. This becomes a problem when one wants to make cell extracts for protein isolation since cell-lysis protocols

invariably release any number of active intracellular proteases that can quickly hydrolyze proteins in the extract. Therefore, an efficient means of inhibiting a wide spectrum of proteases is mandatory for a variety of assays (e.g. western blotting, immunoprecipitation, reporter gene activation, gel shift, enzymatic activity assays etc). For this purpose, identification of naturally occurring inhibitors and design of multiheaded inhibitors is required.

Inhibitors as active–site titrants

Often one must determine the concentration of active enzyme in a crude, or pure but partially inactive preparation of a proteinase. This is most accurately accomplished using specific 'burst' titrants. However these titrants are available for only certain serine proteinases and require relatively large quantities of protein per assay. If material is precious, then titrations using proteinase inhibitors are suitably employed. Given their broad specificity for cysteine proteinases and fast reacting mechanism the *trans* epoxysuccinyl peptides are almost ideal active site titrants. The general serine proteinase-selective inhibitors DFP (di-isopropylfluorophosphate), PMSF (phenylmethylsulphonyl fluoride) and 3,4-DCI (3,4-dichloroisocoumarin) are too unstable in aqueous solutions to be used as active-site titrants. The protein inhibitors, however, can be useful for this purpose.

Inhibitors as anti-cancer agents

One of the diverse biochemical functions reported for proteinase inhibitors is inhibition of growth of transformed cells [116-118]. There is accumulated evidence that consumption of seeds containing protease inhibitors lowers the incidence of breast, colon, prostatic, oral and pharyngeal cancers [119-121]. Several studies have been performed which show that the soybean-derived Bowman-Birk inhibitor can prevent carcinogenesis in vivo and malignant transformation *in vitro* [122,123]. Studies have been intensified on the mechanism of anticarcinogenicity exhibited by these inhibitors [124-126]. Increased exposure to dietary protease inhibitors has been shown to protect against some chemically induced animal tumors [127,128] and it has been suggested that protection against cancer may be achieved through this avenue [129]. Osowole *et al.* [130] have also described the influence of pigeonpea protease inhibitor on the genotoxicity of aflatoxin B_1, a potent animal hepatocarcinogen [131] in *E.coli* PQ37.

Possible role of proteinase inhibitors is also implicated in malarial treatment following a report by Rockett *et al.* [132] on inhibition of intraerythrocytic development of *Plasmodium falciparum.*

PIs as novel anti-fungal agents

Proteinase inhibitors have also been implicated to play a role in the plant's natural defence towards fungal infections [133], further augmenting the potential uses of these insecticidal genes [134-136].

Plant cystatins are active against animal and plant viruses

Much attention has recently been focused on the antiviral activities of various proteinase inhibitors, because many viruses require proteolytic cleavage, which can be specifically inhibited by proteinase inhibitors, to become mature and infectious [137]. Bjorck *et al.* reported that human cystatin C, a thiol proteinase inhibitor, blocks replication of herpes simplex virus (HSV) *in vitro* [138], suggesting an important role of thiol proteinase in the maturation process of the virus. In contrast, a serine-type proteinase is encoded in the virus genome of HSV, that is implicated in the assembly of viral capsids [139,140]. Oryzacystatins I and II (OC-I and OC-II), which occur in rice seed, are the first well-defined cystatins of plant origin that exert potent anti-viral effect against HSV type 1 *in vitro* as well as *in vivo* [141]. OC-I, and OC-II also effectively inhibit the replication of poliovirus *in vitro*, albeit to a lesser extent [142]. Besides this, Vonderfecht *et al.* [143] have also reported the efficacy of PIs ininhibition of retroviral growth *in vitro* and *in vivo* implying a role of PIs in treatment of retroviral diseases like AIDS. Trypsin inhibitors from *Phaseolus lunatus* and *Glycine max*, gossypol and alkaloids from *Corydalis yanhusuo* were able to inhibit HIV-1 reverse transcriptase [144].

Constitutive expression of a rice cysteine proteinase inhibitor gene was found to induce resistance against two important potyviruses, tobacco etch virus (TEV) and potato virus Y (PVY), in transgenic tobacco plants. Tobacco lines expressing the foreign gene at varying levels were examined for resistance against TEV and PVY infection and a clear, distinct correlation was observed between the oryzacystatin message, inhibition of papain (a cysteine proteinase), and resistance to TEV and PVY in all lines [145]. Certain

soybean cultivars are also resistant to Soybean mosaic virus owing to the presence of proteinase inhibitor genes [146].

PIs are also involved in regulation of PCD in plants

A novel role for proteinase inhibitor genes as modulators of programmed cell death (PCD) in plants has been proposed by Solomon *et al.* [147]. They have demonstrated that the ectopic expression of cystatin genes inhibited the induced cysteine protease activity and blocked PCD triggered indirectly by an avirulent strain of *Pseudomonas syringae* pv *glycinea* or directly by oxidative stress. In plants, programmed cell death has been implicated in xylogenesis [148-150], in some forms of senescence, and more importantly in the hypersensitive response to pathogens and environmental stresses [151,152]. This suggests that besides directly targeting pathogens, proteinase inhibitors also indirectly protect the plant from PCD as induced by such pathogen infestations.

Other evidence comes from studies on *AtCYS1*, a cystatin from *Arabidopsis thaliana* which when constitutively expressed in roots and in developing siliques of *A. thaliana*; it is strongly induced in leaves by wounding, by challenge with avirulent pathogens and by nitric oxide (NO). This overexpression of *AtCYS1* blocks cell death activated by either avirulent pathogens or by oxidative and nitrosative stress in both *A. thaliana* suspension cultured cells and in transgenic tobacco plants [153].

PIs are effective against nematodes and other pathogens

Plant proteinase inhibitors are known to confer natural as well as engineered protection against nematode attack [154-157]. Several transgenic approaches to crop improvement have the potential to enhance crop resistance to nematodes [158]. Currently, an anti-feedant strategy, involving expression of proteinase inhibitors (PIs), offers the most advanced approach for nematode control. Cysteine PIs (cystatins) have been demonstrated as the most effective defense against the parasitic stage of *Rotylenchulus reniformis* [159] where the engineered rice cystatin (OC-IDD86) reduced female fecundity and density when expressed in *Arabidopsis* under the control of CaMV35S promoter. Similarly, modified rice cystatin, OC-I delta D86, when expressed as a transgene in *Arabidopsis thaliana*, has a profound effect on the size and fecundity of females for both *Heterodera schachtii* (beet-cyst nematode) and *Meloidogyne incognita*

(root-knot nematode) [160]. The nematode gut cysteine proteinase activity was lost (anti-feedant defense) and no females of either species achieved the minimum size required for egg production. Another study [161] revealed that although the transgenic plants expressing protease inhibitors have enhanced levels of resistance to potato cyst-nematodes (PCN), *Globodera pallida* and *G. rostochiensis*, there was negligible effect on non-target herbivorous insect *Eupterya aurata*; suggesting minimal risk factors involved.

Oryzacystatins I and II are known to form complexes with cysteine proteinases from crude extracts of two-spotted spider mite, a plant pest [162], thus suggesting a possible role against mites as well. Transgenic crop plants expressing phytocystatins could also be used to suppress the growth rates of slug populations in the field as suggested by studies on a transgenic Arabidopsis leaf tissue expressing a modified oryzacystatin that exhibited resistance towards the field slug *Deroceras reticulatum* (Muller) [163].

1.12. PLANT GENETIC ENGINEERING

With the development of disease-, insect- and drought-resistant crops, genetic engineering has addressed atleast some of the environmental problems associated with conventional agriculture. With the increasing demand for food for the burgeoning human population world-wide and decreasing cultivable land, it appears that plant genetic engineering has to be adopted for maximum benefits in a minimum input. The continual need to increase food production necessitates the development and application of novel biotechnologies to enable the provision of improved crop varieties in a timely and cost-effective way. A milestone in this field was the introduction of Bt entomotoxic proteins into plants. Despite the success of this technology, there is need for development of alternative strategies of phytoprotection. Biotechnology offers sustainable solutions to the problem of pests, pathogens and plant parasitic nematodes in the form of other insecticidal protein genes. A variety of genes, besides *Bacillus thuringiensis* (Bt) toxins that are now available for genetic engineering for pest resistance are genes for vegetative inseciticidal proteins (VIPs), proteinase inhibitors (PIs), α-amylase inhibitors (AIs) and plant lectins. Crop protection by means of PI genes is an important component of Integrated Pest Management (IPM) programmes that comprise a combination of control strategies including the judicious use of pesticides, crop rotation, field sanitation and

above all exploitation of inherently resistant plant varieties. Losses due to pests have to be minimized and development of transgenic, insect- and pest-resistant crop varieties through proteinase inhibitor genes will make a promising contribution towards maximising yields.

PI genes: The Advantage

Improved and extended genetic crop resistance is usually seen as a foremost possibility in the current scenario of preventing agricultural losses due to insects and diseases. At present, the main strategy to develop insect- resistant plants via genetic engineering is based on the use of *Bacillus thuringiensis* (Bt) toxin genes. Indeed, the Cry proteins (crystal proteins or endotoxins) cumulate several advantages such as a high specificity, a short life in the environment, and a high and fast toxic activity [164-166]. There are also significant environmental benefits such as absence of pesticide drift, absence of residual pesticide in soil and absence of effects on non-target species. Since the toxin is expressed throughout its tissues, the transgenic plant effectively controls root pests, stem and fruit borers and sucking insects unlike the conventional Bt sprays that could only protect the plant surface [164,167].

However, there are limitations to the use of transgenic *Bt* plants as well. Increased persistence of the *Bt* toxin within the plant throughout the growing season selects intensely for insect resistance [168]. Also, the range of insects which can be controlled by *Bt* toxins are relatively narrow. Moreover, *Bt* toxins have a fairly complex mode of action [169,170]. No *Bt* toxin with adequate aphid toxicity has been described even as aphids continually cause significant crop losses. In order to combat against the broad variety of damaging insects and pests; plants need to strengthen their defense arsenal. Hence, another approach to slowing down insect growth is to use genes that encode for natural defensive compounds that are abundantly found in seeds, namely proteinase and α-amylase inhibitors. Use of PI genes gains importance especially in view of reports on proteinase-mediated insect resistance to *Bacillus thuringiensis* toxins [171].

Moreover, since proteinase inhibitor genes are primary gene products, they are excellent candidates for engineering pest resistance into plants [172]. Inhibitor genes of plant origin are particularly promising. This was first demonstrated by Hilder *et al.* [173] by transferring trypsin inhibitor gene from *Vigna unguiculata* to tobacco, which

conferred resistance to wide range of insect pests including Lepidopterans, such as *Heliothis* and *Spodoptera*, Coleopterans such as *Diabrotica*, *Anthonomnous* and orthoptera such as Locusts. Following these early reports on the success of genetically engineered resistance in plants, several other PIs from diverse plant sources have been studied.

The availability of diverse genes from different plant sources is in itself an advantage as two or more genes can be transferred in combination (with different physiological targets) [174]. Proteinase inhibitors are also reportedly active against nematodes, viral, bacterial and fungal pathogens; thus they may serve to have a cumulative protective effect on plants. Further, there is no evidence that proteinase inhibitors have toxic or deleterious effects on mammals. These advantages make protease inhibitors an ideal choice to be used in developing transgenic crops resistant to insect pests. Table-III lists some successful transformations of plants incorporating the PI or a-amylase inhibitor genes from various sources that duly confer resistance towards insects, pests and pathogens.

To overcome protease inhibitor resistance of insects; it is now necessary to further develop protease inhibitors that are broadly active against all proteases that insects use for digestion. The objective is achieved by:

1) selecting second generation protease inhibitors (novel PIs) from novel sources (unrelated organisms, synthetic libraries or insect haemolymph) with maximum effectiveness against the selected target pests; introducing novel PIs into important crop plants and demonstrating, in the field or greenhouse, that a substantial reduction in the application of insecticides can be achieved.

2) Use of bifunctional α-amylase/trypsin inhibitors is of particular practical interest since transgenic plants expressing a molecule which inhibited both amylases and proteases of pests would be highly protected. The presence of both the activities would also help to minimise the likelihood of the appearance of resistant pest strains.

3) Another approach may be to express two or more PIs as a fusion protein. Expression of a cystatin and a serine PI in this way has been found to be successful against certain nematode pathogens [174].

4) PI genes in conjunction with Bt toxin genes: Studies on the insecticidal activity of transgenic tobacco plants expressing both *Bacillus thuringiensis* insecticidal protein and cowpea trypin inhibitor (CpTI) genes against cotton bollworm (*Helicoverpa armigera*) revealed that it was more effective compared to transgenic tobacco expressing Bt insecticidal protein gene alone. Besides the enhancement of insecticidal efficacy, insect adaptation to transgenic Bt crops is also delayed [215].

TABLE-III

Transgenic plants carrying protein proteinase inhibitor genes in defense against insects, pests and pathogens

SOURCE GENE	TRANSFORMED PLANT	DEFENSE AGAINST	REFERENCE
Proteinase inhibitor genes			
Cowpea trypsin inhibitor(CpTI)	tobacco	*Heliothis virescens* larvae	[175]
	tobacco	*Spodoptera litura*	[176]
	cotton	*Helicoverpa armigera*	[177]
	rice	Rice stem borers: *Chilo suppressalis* and *Sesamia inferens*	[178]
	potato	Tomato moth: *Lacanobia oleracae*	[179]
	strawberry	Vine weevil: *Otiorynchus sulcatus* F.	[180]
	cabbage	*P.rapae*	[181]
	pigeonpea	*Helicoverpa armigera*	[182]
CpTi and snowdrop lectin	sweet potato	*Cyclas formicarius*	[183]
	'Jewel'sweet potato	West Indian sweet potato weevil: *Euscepes postfaciatus*	[184]
Soybean serine-proteinase inhibitor (C-II)	potato/tobacco	Coleoptera/Lepidoptera	[111]
Soybean (Kunitz) trypsin inhibitor	tobacco	*Spodoptera litura* larvae	[185]
	rice	Brown planthopper: *Nilaparavata lugens* Stal	[186]
Soybean Kunitz trypsin inhibitor (SKTI-4)	sweet potato	*Cyclas* spp.	[187]
Soybean Kunitz, C-II and PI-IV inhibitor	potato/tobacco	*Spodoptera littoralis*	[110]
Trypsin inhibitor from *Vigna unguiculata*	tobacco	*Heliothis* *Spodoptera* *Diabrotica* *Anthonomnous* *Locusts*	[165]

.....continued

Tomato proteinase inhibitors I&II	tobacco	*Manduca sexta* larvae	[188]
Tomato proteinase inhibitor I	nightshade	---	[189]
	tobacco	---	[189]
	alfalfa	---	[189]
Proteinase inhibitor SaPIN2a	lettuce	----	[190]
Potato inhibitor II gene	tobacco	*Chrysodeisus eriosoma*	[191]
	rice	Pink stem borer: *Sesamia inferens*	[192]
	sugarcane	Sugarcane grubs: *Antitrogus consanguineus*	[193]
Sweet potato (*Ipomea batatas*) trypsin inhibitor	tobacco	*Spodoptera litura*	[194]
Trypsin inhibitor from barley(CMe)	indica and japonica rice	Rice weevil: *Sitophilus oryzae*	[195]
	barley	*Agrotis ipsilon*	[196]
	tobacco	Lepidoptera	[196]
	wheat	*Spodoptera lituralis*	[197]
Mustard trypsin inhibitor-2	----	*Spodoptera littoralis* larvae	[198]
Mustard trypsin inhibitor (MTI-2)	tobacco	*Plutella xylostella* (L.)	[199]
	Arabidopsis	*Mamestra brassicae* (L.)	[199]
	oilseed rape	*Spodoptera littoralis* (Boisduval)	[199]
Oryzacystatin I	poplar	*Chrysomela tremulae* (Coleoptera: Chrysomelidae)	[200]
	potato	Peach potato aphid: *Myzus persicae*	[201]
	potato	Colorado potato beetle larvae *Leptinotarsa decemlineata*	[202]
	oilseed rape	cabbage seed weevil (Coleoptera: Curculionidae)	[203]
	oilseed rape	*Myzus persicae*	[105]
Corn cystatin	rice	Maize grain weevil: *Sitophilus zeamais*	[204]

.....continued

Nicotiana alata	tobacco	*Helicoverpa punctigera*	[205]
protease inhibitor	pea	*Plutella xylostella*	[206]
Arabidopsis thaliana	white poplar	Chrysomelid beetle:	
cysteine proteinase inhibitor		(*Populus alba* L.) *Chrysomela populi*	[207]

PI genes against other pathogens (Virus/Nematode/etc.)

Modified oryzacystatin	*Arabidopsis thaliana* leaf tissue	Field Slug: *Derocerus reticulatum*	[163]
	Arabidopsis	Beet-cyst nematode: *Heterodera schachtii*	[160]
		Root-knot nematode: *Meloidogyne incognita*	[160]
Cysteine PI	tobacco	Potyviruses	[208]
Oryzacystatin	transgenic hairy roots	*Globodera pallida*	[209]
Oryzacystatin I and II	alfalfa	root-lesion nematode	[210]
Oryzacystatin I delta D86	rice	nematode resistance	
		Meloidogyne incognita	[211]
		Rotylenchulus reniformis	[159]

α-amylase inhibitors in plant defense

Bean α-amylase inhibitor I	azuki bean	bruchid resistance	[212]
	pea	pea weevil:*Bruchus piorum*	[213]
	pea	Bruchid beetle	[214]

Materials & Methods

2. MATERIALS & METHODS

2.1 MATERIALS

The pulses *Cajanus cajan* (Pigeonpea; decorticated split cotyledons) and *Phaseolus aureus* (Mung bean) were procured from the local market. Trypsin (3x crystallized), α-chymotrypsin (3x crystallized), Dithiothreitol, Hammersten casein and dimethylsulfoxide were purchased from Sisco Research Laboratories, India. N-Acetyl-L-tryptophanamide (NATA), N-Acetyl L-tyrosine ethyl ester, N_{α}-Benzoyl-L-Arg-p-nitroanilide (BAPNA) (Lot 37F0833), N-Glutaryl-Gly-Gly-Phe β–Naphthylamide (Lot 34F0402), DEAE cellulose (Lot 113H0367), TPCK treated trypsin (EC 3.4.21.4) (Lot 11K7264), bovine serum albumin and iodoacetamide were obtained from Sigma Chemical Co. (St. Louis, MO). N-Bromosuccinimide was a product of Qualigens Fine Chemicals, India. 5,5' Dithiobis nitrobenzoic acid (DTNB) was obtained from Sisco Research Laboratories, India. Sodium dodecyl sulfate (SDS), sodium deoxycholate (DOC), 3-[(3-cholamidopropy) dimethylammonio]-1-propane sulfonate (CHAPS) and 3,3,3,3',3',3'-hexafluoro-2-propanol (HFIP) were procured from Sigma Chemical Co., USA.

2.2 METHODS

2.2.1 Estimation of protein concentration

Protein concentration was routinely determined by the method of Lowry *et al.* [216] using bovine serum albumin (BSA) as standard.

2.2.2 pH measurements

pH measurements were carried out on an ELICO digital pH meter, model LI 610. The least count of the pH meter was 0.01pH unit. The pH meter was routinely calibrated at room temperature with either 0.05 M potassium hydrogen phthalate buffer, pH 4.0 in the acidic range or .01 M sodium tetra borate buffer, pH 9.2 in the alkaline range.

2.2.3 Optical measurements

Hitachi U-1500 UV-VIS spectrophotometer was used for light absorption measurements in the ultraviolet as well as visible region using quartz cells of 1 cm path

length. Absorption measurements in visible range were also made on AIMIL Photochem-8 colorimeter using glass cuvettes of 1cm pathlength.

2.2.4 Purification of Inhibitors

Pigeon pea (C.cajan):

20 grams of *C. cajan* pulse was soaked overnight in 100ml, 0.02M Tris- HCl buffer, pH 8.2 containing 2mM $CaCl_2$. Thereafter it was homogenized in a blender for 2-3 min at moderate speed. The crude extract was filtered through cheesecloth to remove the coarse residual matter. It was then centrifuged at 8000 rpm for 45 min at 4°C. The clear supernatant thus obtained was defatted with 0.1 M acetic acid (final pH of solution = 4.0). After overnight stirring, the extract was subsequently centrifuged at 8000 rpm for 45 min at 4°C. The supernatant was adjusted to pH 7.0 with liquor ammonia and raised to 50% saturation with ammonium sulphate. The precipitate was collected by centrifugation at 8000 rpm for 45 min, 4°C; dissolved in minimum amount of 2mM $CaCl_2$ -20mM Tris-HCl (pH 8.2) and dialyzed against the same buffer for 24 hrs.

The dialyzed sample (10ml) was applied to a DEAE cellulose column (2.2 cm x 9.9 cm) equilibrated with 2mM $CaCl_2$ -20mM Tris- HCl (pH 8.2) and the bound protein eluted with the same buffer containing 0.2M NaCl. The fractions containing inhibitory activities against trypsin and α-chymotrypsin were pooled and used as the proteinase inhibitor (PI).

Mung bean (P.aureus):

Finely ground powder of pulse *P. aureus* was soaked overnight in 20mM sodium phosphate buffer, pH 7.4. Thereafter it was homogenized in a blender for 2-3 min at moderate speed. The crude extract was filtered through cheesecloth to remove the coarse residual matter. It was then centrifuged at 8000 rpm for 45 min at 4°C. The clear supernatant thus obtained was defatted with 0.1 M acetic acid (final pH of solution = 4.0) and kept overnight and allowed to precipitate, the extract was subsequently centrifuged at 8000 rpm for 45 min at 4°C and the precipitate discarded. The supernatant was adjusted to pH 7.0 with liquor ammonia and raised to 50% saturation with ammonium sulfate. The precipitate thus obtained was removed by centrifugation at 8000 rpm for 45 min, 4°C; and the supernatant was further subjected to 50-70% salt precipitation. After overnight

incubation the precipitate was collected by centrifugation at 8000 rpm for 45 min, 4°C; dissolved in minimum amount of 20 mM sodium phosphate buffer and dialyzed against 10mM Tris-HCl buffer, pH 8.2 for 24 hrs. The dialyzed sample was applied to a DEAE cellulose column (2.2 cm x 9.9 cm) for further purification. It was subsequently washed with 10 volumes of equilibration buffer (10mM Tris-HCl buffer, pH 8.2) to remove the unbound proteins. The bound proteins were eluted with a salt gradient of 0-1.0M NaCl in the equilibration buffer. Fractions (3 ml) were collected and monitored for protein by measuring the absorbance at 280 nm. The fractions were also tested for the inhibitory activity against trypsin. Fractions showing inhibitory activity were then pooled. Fractions showing inhibitory activity were then pooled and rechromatographed on DEAE-cellulose column. The active fractions obtained after elution with 0.15M NaCl were pooled and further purified on a Trypsin-Sepharose (2.2 cm x 10 cm) column. The column was equilibrated with 20mM sodium phosphate buffer, pH 7.0 and bound protein eluted by lowering the pH with 20mM glycine-HCl buffer, pH2.0.

2.2.5 Determination of Molecular weight

For the determination of *C.cajan* PI molecular weight, SDS-PAGE was performed on 15% polyacrylamide slab gel under reducing conditions by the method of Laemmli [218]. Proteins were detected by staining the gel with 0.1% Coomassie brilliant blue R-250. The molecular weight of the *P.aureus* inhibitor was determined by SDS polyacrylamide gel electrophoresis of 10μg of protein on a 12% polyacrylamide gel with 2.5mA per well. The gel was stained by performing silver staining [219]. Molecular weight markers used were bovine α-lactalbumin (14kDa); soybean trypsin inhibitor (20kDa); carbonic anhydrase (30kDa); ovalbumin (43kDa)and serum albumin (67kDa).

2.2.6 Enzyme Inhibion Assay

Inhibtory activity against the trypsin was checked by the method of Erlanger *et al* [220]. 0.1 ml of enzyme (0.25 mg/ml) was pre incubated with increasing amounts of inhibitor (molar ratio varied from 0-2) for 10 min, followed by the addition of 5 ml of 1mM substrate solution (43.5 mg of Nα-Benzoyl L-Arg p-nitroanilide dissolved in 0.5 ml of dimethylsulfoxide and volume adjusted to 100ml with buffer). The reaction was allowed to proceed for 15 min at 37 °C in a thermostat and stopped by the addition of 1 ml of 30% glacial acetic acid. Suitable controls of enzyme alone in the absence of

inhibitor were also made and measured for optimum enzyme activity. The residual enzyme activity was measured by monitoring the liberation of p-nitroaniline at 410nm on a Hitachi U-1500 spectrophotometer.

In case of α-chymotrypsin, 1 mM N-Acetyl L-tyrosine ethyl ester solution was used as substrate. Fixed amount of enzyme (0.4 mg/mL) was preincubated with inhibitor as described earlier and measured for residual enzyme activity in the absence and presence of increasing amounts of *P. aureus* inhibitor. The solutions were kept at 37°C for 15 min. The extent of substrate hydrolysis was monitored by measuring the decrease in absorbance at 237 nm [221].

Inhibitory activity towards an acid protease, pepsin was measured by carrying out the Worthington pepsin assay (based on the method of Ansen [222]) using hemoglobin as substrate in the presence of inhibitors. 2% (w/v) hemoglobin solution (prepared in 6M urea; sodium phosphate buffer, pH 7.5) was equilibrated to 37°C. A fixed amount of enzyme (0.5ml) in the absence and presence of inhibitor (also equilibrated to 37°C) was mixed with 2.5 ml of substrate solution and allowed to incubate at 37°C for exactly 10 min. The reaction was subsequently stopped by the addition of 1ml of 15% trichloroacetic acid (TCA). After centrifugation at 10,000 rpm at 4°C for 15 min, the clarified TCA soluble fractions were read at 275 nm.

Inhibitory activity towards cysteine proteinases was also assessed using Hammersten casein as substrate [223]. 2%(w/v) casein was prepared in 100mM sodium phosphate buffer, pH 7.5 by gently heating to 80-90°C for 10 min. with constant stirring. Fixed amount of enzyme (stem bromelain) was incubated with appropriate concentrations of inhibitor at 37°C for 5 min. followed by the addition of 2 ml of substrate. The reaction was allowed to proceed at 37°C for 10 min. and stopped by the addition of 1ml 15% TCA solution. Appropriate controls without inhibitor were also prepared. After centrifugation to remove the precipitate, absorbance was read at 280 nm.

For the measurement of α-amylase inhibitory activity, the method of Bernfeld [224] was followed. A fixed concentration of enzyme (0.2 μg) was preincubated with increasing concentrations of *P. aureus* inhibitor (Total volume, 1.5 ml) at 37°C for 20 min. (in 30μmol sodium phosphate buffer, pH 6.9 containing 10.5μmol NaCl) followed by the addition of 0.5ml, 1% starch. Substrate hydrolysis was allowed to proceed for 5

min at 37°C after which the reaction was stopped by the addition of 1ml DNS reagent. Samples were then kept in a boiling water bath for 10 min, allowed to cool and diluted with 5ml distilled water. Appropriate controls were also prepared in the absence of inhibitor. Inhibitory activity was assessed by the decrease in absorbance values at 550nm.

2.2.7 Stability Studies

pH

Effect of pH on the inhibitory activity of *C. cajan* and *P. aureus* proteinase inhibitors was examined at different pH values by preincubating the inhibitor (0.25 mg/ml) in respective buffer solutions at 37.0 ° C for 30 min. Required aliquots were pipetted into the assay mixture containing enzyme and reaction was started by the addition of substrate. The pH range examined was 2.0 –12.0. Buffers used were 0.01M Glycine-HCl (pH 2.0-3.0); 0.01M sodium acetate (pH 4.0-6.0); 0.01M sodium phosphate (pH 7.0-8.0); 0.01M Glycine-NaOH (pH 9.0-12.0).

Temperature

To measure the thermal stability of the inhibitory activity, the inhibitor solutions (0.25 mg/ml) were heated for 1h in a water bath at various temperatures (30-90°C) and then cooled in ice. The samples were then brought back to 37°C for measurement of residual inhibitory activity. Alternatively, inhibitor samples were subjected to dry heat at fixed temperatures in a thermostat and checked for residual inhibitor activity.

Reducing agent (Dithiothreitol)

The *C. cajan* inhibitor (0.5mg/ml) in 2mM $CaCl_2$ -20 mM Tris -HCl (pH 8.2) was treated with DTT (2mM and 10mM final concentrations) for 60 min. at room temperature and the reaction stopped with 2 fold molar excess of iodoacetamide. After stirring for 30min at 4°C, the reduced samples were dialyzed against the same buffer and used for activity measurements as well as quenching experiments.

Proteolysis

Stability of the *P. aureus* inhibitor towards tryptic cleavage with respect to time was examined by incubating the inhibitors with 3-fold molar excess of enzyme for varying time periods (10-60 min) and assaying for residual inhibitory activity.

2.2.8 Estimation of Trp, Tyr and Cys

Tryptophan

Tryptophan content was assessed by the method of Goodwin and Morton [225]. The absorption spectrum of *C.cajan* PI was measured in the wavelength range 300-220 nm in the presence of 0.1M NaOH. The formula used was: $w = (A_{280} - x.\varepsilon_y)/ (\varepsilon_w - \varepsilon_y)$ where w was estimated tryptophan content in moles per liter; A_{280} was absorbance at 280 nm; ε_y and ε_w were molar extinction coefficients of tyrosine and tryptophan in 0.1 M alkali at 280 nm (ε_y = 1576 and ε_w = 5225).

Tryptophan content was also estimated by the method of Spande and Witkop [226]. The initial optical density of the *C.cajan* PI or *P.aureus* proteinase/amylase inhibitor in 8M urea at 280 nm was recorded and further decrease in optical density by the addition of a fixed aliquot (10µl) of 10 mM stock solution of N-Bromosuccinimide. The addition was continued in this stepwise fashion until further increments lead to no further decrease in optical density, with due allowance for the small decrease expected from dilution. The minimum optical density is recorded and corrected for the volume increase due to the added reagent. The reagent itself and succinimide display negligible absorption at 280 nm.

Tyrosine

Tyrosine content was estimated by the ionization of phenolic hydroxyl groups. The titration of the phenolic groups of the isolated inhibitors was carried out spectrophotometrically [227,228] at 30°C. Two identical solutions of inhibitor (1mg/ml) at pH 6.0 and pH 13.0 were prepared and an alkaline difference spectrum of the inhibitor was recorded in the wavelength region 236-320 nm. Extreme care was taken to ensure identical protein concentration in the two samples.

Cysteine

The total sulfhydryl content of the purified enzyme were measured by the method of Habeeb [229] using DTNB in which the release of nitrothiobenzoate anion was measured by following the increase in absorbance at 410 nm. The protein preparation was denatured by 6M Gdn.HCl and reduced by 0.025M β-mercaptoethanol. After 2 hours, the protein was precipitated with 5% trichloroacetic acid and precipitate resuspended in 8M urea. Subsequently 2% SDS was added and the solution titrated against DTNB.

Molar extinction coefficient was calculated by the proposed equation of Pace et al. [230]:

$$\varepsilon_{(280)}(M^{-1}cm^{-1}) = (\#Trp)(5,500) + (\#Tyr)(1,490) + (\#cystine)(125) \quad (1)$$

2.2.9 Fluorescence quenching studies

Fluorescence quenching measurements were performed on a Shimadzu RF 540 spectrofluorometer equipped with a data recorder DR-3 using an excitation wavelength of 280 nm and emission wavelength range of 300-400nm. *C. cajan* PI samples, native as well as reduced and N-acetyl-L-tryptophanamide (NATA) were titrated with 2M acrylamide and 1M potassium iodide (containing 0.1mM sodium thiosulphate to prevent oxidation and formation of I_3^-). Relative fluorescence intensity was recorded with progressive addition of 10µl aliquots of the quenchers. Since acrylamide had intrinsic absorption at 280nm the inner filter effect was corrected using the equation:

$$F_{corr} = F_{obs} \cdot 10^{A/2} \quad (2)$$

where A is the absorbance of the sample at 280nm by the addition of acrylamide [231]. The contribution of collisional quenching to the deactivation rate of an excited state fluorophore is given by the Stern-Volmer equation:

$$F_0/F = 1 + K_{SV}[Q] \quad (3)$$

where F_0 and F are the fluorescence intensities in the absence and presence of a quencher at a molar concentration of [Q], and K_{SV} is the Stern-Volmer constant which is the product of a collisional quenching rate constant and the excited state lifetime of the fluorophore in the absence of quencher. In a heterogenous population of fluorophores, like tryptophan residues in a protein, the linear relationship between F_0/F and [Q] is generally not obeyed. In such species it is assumed that energy transfer between accessible and inaccessible fluorophores is negligible and that all fluorophores have identical absorptivities. The fluorescence quenching is described by the modified Stern-Volmer equation :

$$\frac{F_o}{F_o - F} = \frac{1}{f_a K_{sv}} \cdot \frac{1}{[Q]} + \frac{1}{f_a} \qquad (4)$$

where f_a is the maximum fraction of the protein fluorescence accessible to quencher.

2.2.10 Circular Dichroism (CD) conformational studies

Circular dichroism (CD) measurements were made on a Jasco J-720 spectropolarimeter thermostatted with a Neslab water bath precalibrated with d-10-camphorsulfonic acid. The measurements were made at 25°C using quartz cells of 1mm pathlength in the far-UV region and 1cm pathlength in the near-UV region. The pathlenghts of the cell and protein concentration (0.2-0.5 mg/ml) were chosen to optimize the measuring conditions. Each spectrum was an average of two scans. Results are expressed in terms of $[\theta]_{MRW}$ [232] where

$$[\theta]_{MRW} = \frac{\theta x\ 100 \times MRW}{c \times d} \qquad (5)$$

where θ is the measured ellipticity in degrees, c is the protein concentration in mg/ml, d is the pathlength in cm, and MRW is the mean residue weight. The mean residue weight used was 110 in all cases.

2.2.11 Inhibition kinetics

The kinetics of association of *Cajanus cajan* and *P. aureus* inhibitors to trypsin and α-chymotrypsin were analysed by measurements of the loss of enzyme activity in the presence of specific chromogenic substrates under pseudo first-order conditions [233]. The enzymes were mixed with a large excess of inhibitor and samples withdrawn at timed intervals to measure the residual enzyme concentration [E]. The initial concentration of PI was varied from 0.1-1.7μM for analyses with trypsin and from 0.08-8.6μM for analyses with α-chymotrypsin and substrate hydrolysis did not exceed 10%. k_{ass} was calculated from the relationship:

$$k_{ass} = k_{obs} / [I]_o \qquad (6)$$

where k_{obs} ,(pseudo first-order rate constant) was obtained from the slope of a plot of ln[E] (ln[residual enzyme activity]) against t (time of sampling) and $[I]_o$ represents the inhibitor concentration.

Inhibition constants, K_i, for the binding of inhibitors to trypsin and α-chymotrypsin were determined from the equilibrium rates of substrate hydrolysis by the enzymes at different inhibitor concentrations. Apparent inhibition constants, $K_{i(app)}$ were obtained by least-squares regression of plots of the ratio between the inhibited and uninhibited rates of substrate hydrolysis against inhibitor concentration as given by

$$v_o/v_i = 1+[I]_o / K_{i(app)} \quad (7)$$

where v_o represents the uninhibited rate and v_i, the inhibited rate of substrate hydrolysis. K_i, the true equilibrium constants were calculated after correction for substrate competition.

2.2.12 N-terminal protein sequencing

The protein sample for sequencing was electrophoresed according to the procedure given by Smith and transferred as a blot onto a PVDF membrane [234]. The N-terminal sequence was determined on a Shimadzu PPSQ-10 automated protein sequencer using Edman degradation. Phanylthiolhaydantoin amino acids (PTH-AA) were detected at 269 nm after separation on a reverse-phase CR7A Chromatopac HPLC column (4.6 mm X 25 cm) from Shimadzu, under isocratic conditions, using 40% acetonitrile, 20mM acetic acid and 0.014% sodium dodecyl sulfate as the mobile phase at a flow rate of 1.0 ml/min at 40°C.

2.2.13 Effect of detergents and hexafluoroisopropanol (HFIP) on conformation and inhibitory activity

The effect of detergents on the conformation and activity of the protein inhibitors was investigated using circular dichroism, fluorescence and enzyme inhibition studies. Detergents used were sodium dodecyl sulfate (SDS; 0-1mM), sodium deoxycholate (SDC; 0-4mM) and CHAPS (0-15mM) in the concentration ranges stated. Hexafluoroisopropanol was added to a fixed amount of inhibitor in the concentration range (0-30%, v/v).

Results & Discussion-I

Cajanus cajan (L.) Millsp.

Syn. ***Cajanus indicus*** **Spreng.**

Leguminosae

Alverja, Bengal bean, Congo pea, Dhal, Gandul, Goongoo pea, Gray pea, Gungo pea, Indian dhal, Indian pea, No-eye pea, Pigeon pea, Pois cajun, Pois d'Angole, Red gram, Yellow dhal

Kingdom:	Plantae
Division:	Magnoliophyta
Class:	Magnoliopsida
Order:	Fabales
Family:	Fabaceae
Genus:	Cajanus
Species:	cajan

3. RESULTS AND DISCUSSION

3.1 *Cajanus cajan* (Pigeon pea)

3.1.1 Purification of *C. cajan* proteinase inhibitor

The purification details of *C. cajan* proteinase inhibitor (PI) are summarized in Table-IV. The procedure yielded 15mg of PI from a 20 gm dry weight of pulse. The elution profile of the PI from DEAE cellulose column is represented in figure 4. No appreciable amounts of tannins have been reported in pigeonpea but they do contain considerable amounts of polyphenolic compounds that inhibit digestive enzymes. Polyphenols constitute about 3-18.3 mg/g of pigeonpea seeds [235,236]. To rule out the contribution of polyphenolic compounds to the inhibitory activity, some purified samples of *C. cajan* PI were passed through a Sephadex G-50 column [237]. No alteration in the inhibitory activity was observed before and after passage through the column suggesting that polyphenolic constituents did not contribute to the inhibitory activity.

SDS- polyacrylamide gel electrophoresis of the eluted *C. cajan* PI under reducing conditions yielded a single band corresponding to a molecular weight of 14,000 ϒ(Fig.5).

3.1.2 Trypsin and chymotrypsin inhibitory activities

The *C. cajan* proteinase inhibitor was found to specifically inhibit trypsin and α-chymotrypsin. Caseinolytic activities of papain and stem bromelain were not inhibited by the inhibitor. Figure 6 depicts the inhibition of amidolytic activity of trypsin and caseinolytic activity of α-chymotrypsin by the *C. cajan* PI. The proteolytic activity of trypsin was reduced sharply at very low concentrations of *C. cajan* PI. Even at [inhibitor]/[enzyme] molar ratio of 0.2; inhibition of enzyme activity was more than 80%. The maximal inhibition of trypsin was 86.9 ± 1.57. In case of α-chymotrypsin almost 50% reduction in activity was observed at [inhibitor]/[enzyme] molar ratio of 0.2. This was followed by a gradual decrease (upto 65% ± 0.35) in the residual enzyme activity. Greater reduction in the activity of trypsin as compared to α-chymotrypsin clearly indicates that the PI is more effective against trypsin.

Table IV

Purification table of *C. cajan* proteinase inhibitor (PI)

Step	Total protein (mg)	Total Inhibitory Activity (IU[a])	Specific Inhibitory Activity (IU/mg)	Purification (fold)	Yield (%)
1. Homogenate	1300	678600	522	1	100
2. Acid-precipitation	800	nd*	---	---	61.5
3. 50% Ammonium sulphate fraction	242	199625.8	824.9	1.58	18.6
4. DEAE – Celluose chromatography	15	14800	986.7	1.89	1.2

[a] **Unit definition:** One trypsin unit = ΔA_{410} of 0.001 per min with BAPNA as substrate at pH 8.2 at 37°C. Reaction volume = 7 ml (1 cm light path). Inhibition thereof = IU

*nd - not determined

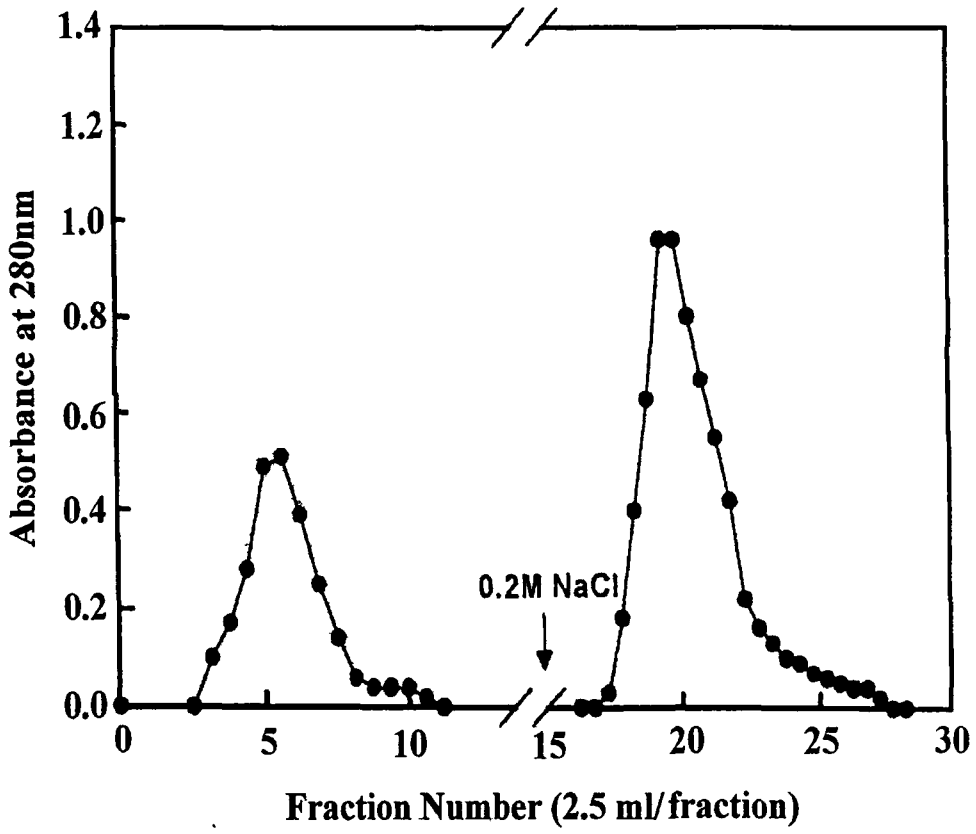


Fig. 4. Elution profile of *C..cajan* PI on a DEAE-Cellulose chromatography column (2.2 cm x 9.9 cm) equilibrated with 2mM $CaCl_2$ - 2mM Tris-HCl buffer (pH 8.2). The PI was eluted batchwise with 0.2M NaCl in the equilibration buffer.

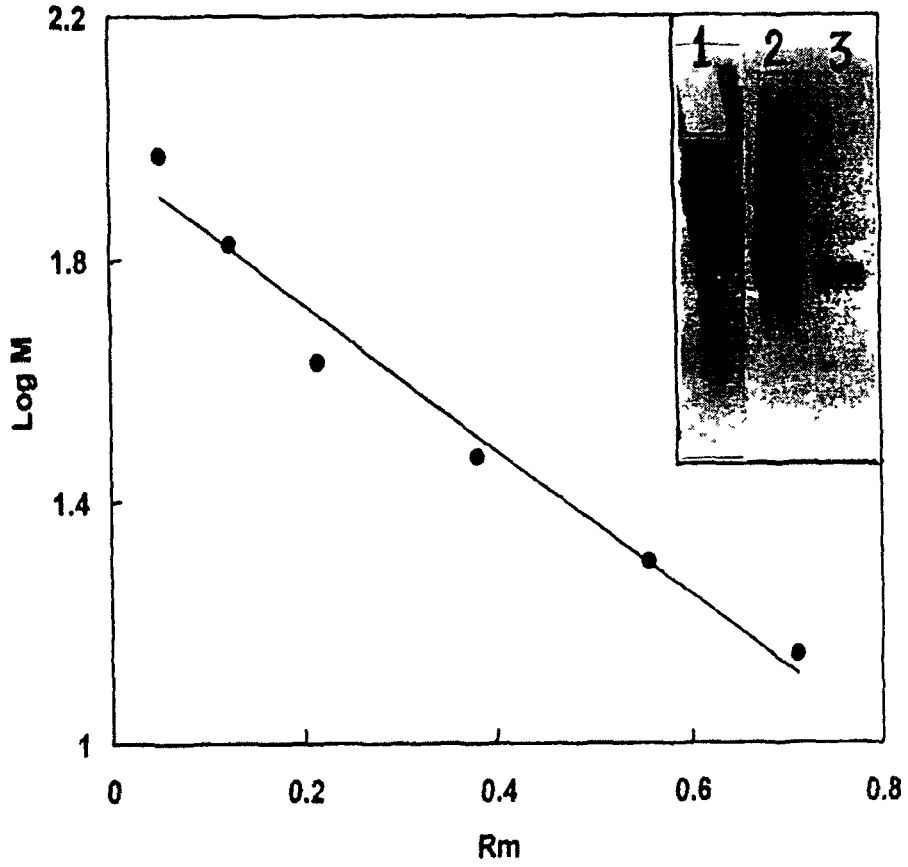


Fig.5. Molecular weight determination by SDS-PAGE: Log M versus relative mobility (Rm) of molecular weight markers: Phosphorylase b (Mr 94,000), Serum albumin (Mr 67,000), Ovalbumin (Mr 30,000), Carbonic anhydrase (Mr 43,000), Soybean trypsin inhibitor (Mr 20,000) and Bovine α- lactalbumin (Mr 14,000). Inset: Electrophoresis profile of the proteinase inhibitor from *C. cajan* (lane 3). Lanes 1 and 2 are crude homogenate and 50% ammonium sulphate fraction respectively.

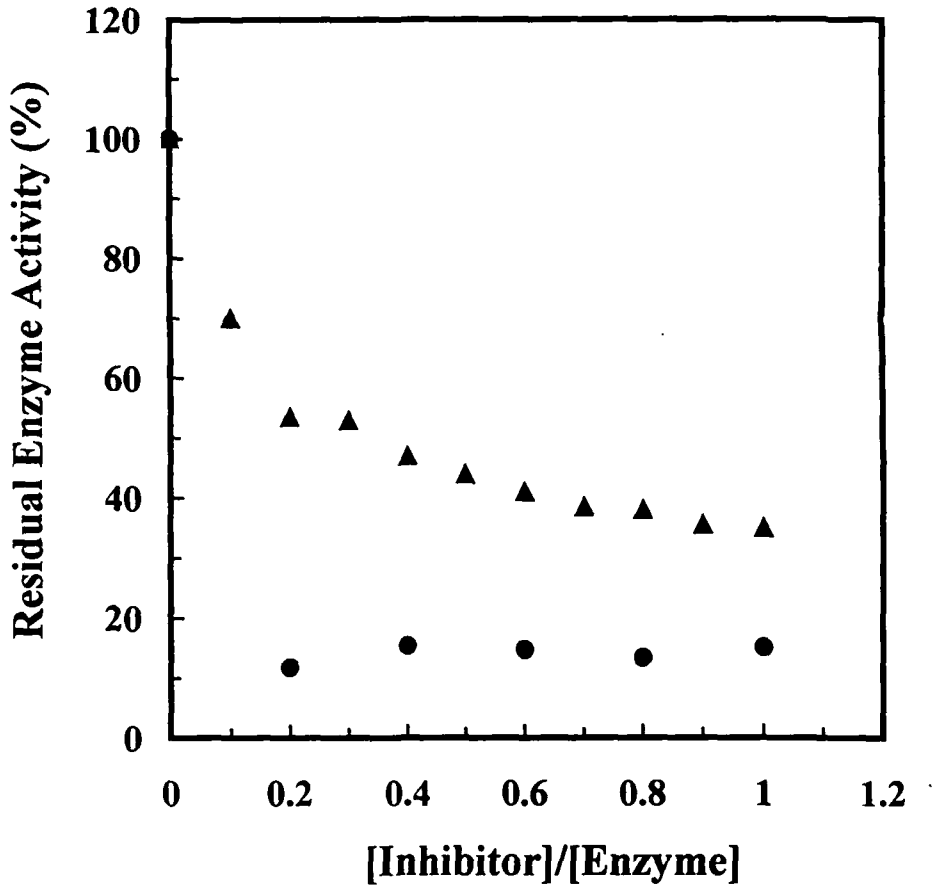


Fig.6. Percent residual enzyme activity for trypsin (●) and α-chymotrypsin (▲) in the presence of varying [*C. cajan* PI]/[Enzyme] molar ratios.

3.1.3 Estimation of tryptophan (Trp), tyrosine (Tyr) and cysteine (Cys)

The number of tryptophans calculated according to the method of Goodwin and Morton were ~2 (Table V). That determined by the method of Spande and Witkop [226] was also ~2. A representative fluorescence emission spectrum of *C. cajan* PI is given in figure 7. In proteins containing all three amino acids viz., tryptophan, tyrosine and phenylalanine; the observed emission is due mainly to tryptophan. Proteins that contain no tryptophan, such as ribonuclease show the emission band due to tyrosine. The fluorescence emission spectra of *C. cajan* PI as shown in the figure clearly indicates an emission wavelength maximum (λmax) at 340 nm that is a characteristic feature of this amino acid [238]. No tryptophan containing protein so far has been shown not to give tryptophan fluorescence. Therefore presence of indole fluorescence can be considered as a good evidence that the protein is containing this amino acid.

The alkaline difference spectrum (Fig. 8) of *C. cajan* PI shows two peaks at 244 nm and 296 nm and an isosbestic point near 272 nm which is characteristic of the ionization of phenolic hydroxyl groups of tyrosine. In order to calculate the number of ionized tyrosyl residues, the maximum change in the molar extinction at 296 nm was measured and divided by 2300 cm^2/mole, the Δε of free tyrosine residue. Estimation of tyrosines by this method yielded 1.8 ionizable tyrosine residues in *C. cajan* PI (Table V).

The sulfhydryl groups in *C. cajan* PI were titrated against DTNB under native as well as denaturing conditions. Under native conditions, only one mole of DTNB reacted per mole of the protein suggesting one free sulfhydryl group. However, under denaturing conditions the absorbance at 410 nm was found to increase with increase in the ratio of [DTNB]/[*C. cajan* PI] and the inflection point in the curve (Fig.9) occurs at 5.0 indicating the presence of a total of ~ 5 sulfhydryl groups. This suggests the presence of atleast two disulfide linkages in *C. cajan* PI.

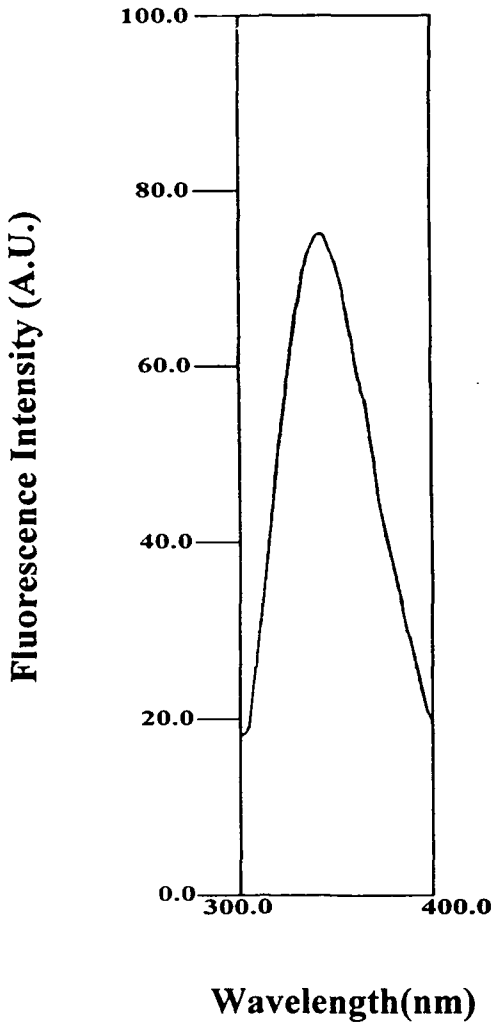


Fig.7. Fluorescence emission spectrum of *C. cajan* PI. λ_{ex} = 292 nm.

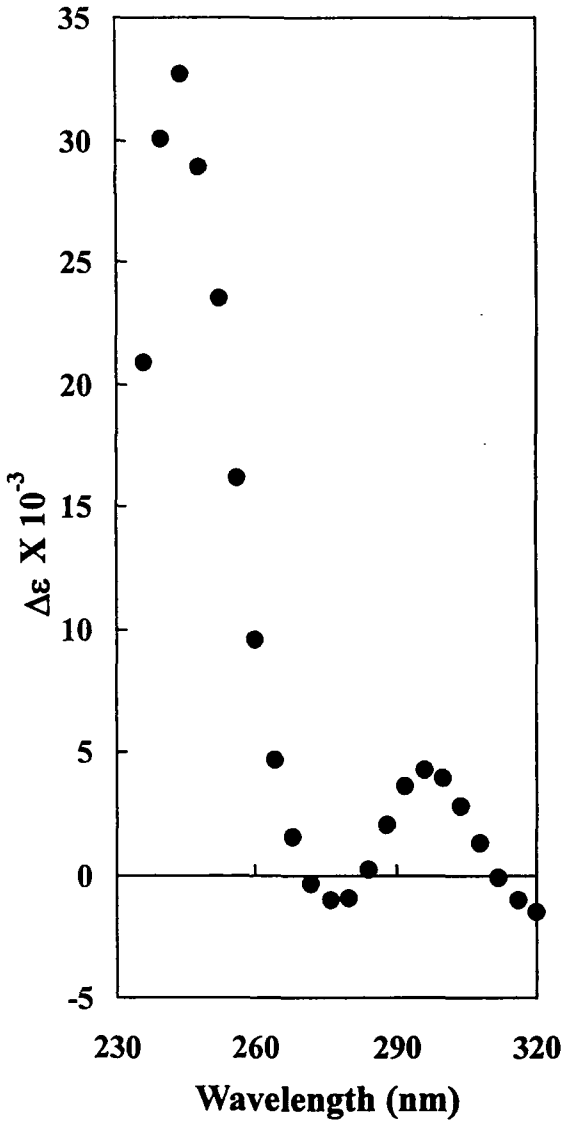


Fig.8. Alkaline difference spectrum of *C. cajan* PI in the wavelength range 320-230 nm.

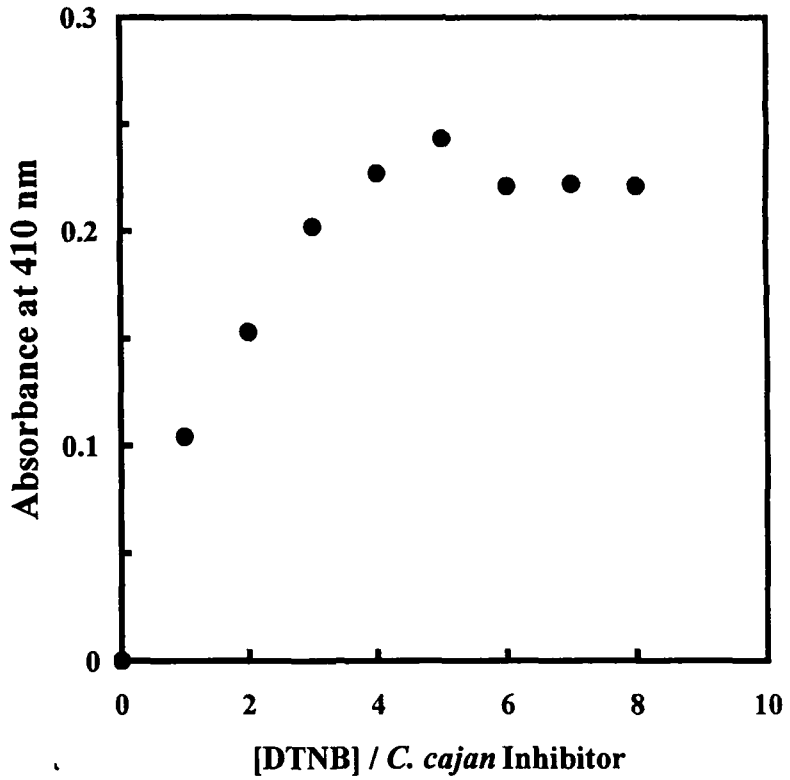


Fig.9. Sulfhydryl estimation. Plot of A_{410} versus [DTNB]/ *C. cajan* inhibitor. Fixed concentration of inhibitor was titrated against increasing molar excess of DTNB.

Table V

Estimation of tryptophan, tyrosine and sulfhydryl content

Amino acid	Estimated number [b]	Method of analysis
Tryptophan (Trp)	2.0 ± 0.49	Goodwin & Morton [225]
	2.1 ± 0.12	Spande & Witkop [226]
Tyrosine (Tyr)	1.8 ± 0.03	Qasim & Salahuddin [227]
Cysteine (Cys)	5.7 ± 0.12	Habeeb [229]

[b] The results are obtained as a mean of three independent observations

3.1.4 Effect of temperature and pH on *C. cajan* PI

The residual inhibitory activity of *C. cajan* PI was measured after pretreatment of the inhibitor at different temperatures for 30min and results are depicted in figure10A. As seen in the figure, the proteinase inhibitor was quite resistant to temperature denaturation. Even after heating the PI at 95°C for 30 min, the residual inhibitory activity was approximately 85%. This is in agreement with an earlier report by Mulimani and Paramjyothi [239] that the application of dry heat to the seeds and meal of red gram was not effective in inactivating the trypsin inhibitory activity (TIA) and chymotrypsin inhibitory activity (CIA).

The effect of pH on the activity profile of trypsin in the absence and presence of *C. cajan* PI was also tested (Fig.10B). The proteinase inhibitor was found to significantly inhibit the amidolytic activity of trypsin in the pH range 6.0 - 10.0 suggesting that the PI was also quite resistant to pH effects in this region.

3.1.5 Effect of reducing agent on activity of *C. cajan* PI

The effect of 2mM dithiothreitol (DTT) treatment on the inhibitory activity of *C. cajan* PI was also checked. Table VI shows that the inhibitory activity of PI decreases upto 50% upon treatment with dithiothreitol for 30 min and is further decreased to 18% upon reduction with DTT for 1hr. This is indicative of the presence of disulfide linkages important for the structure and inhibitory activity of PI.

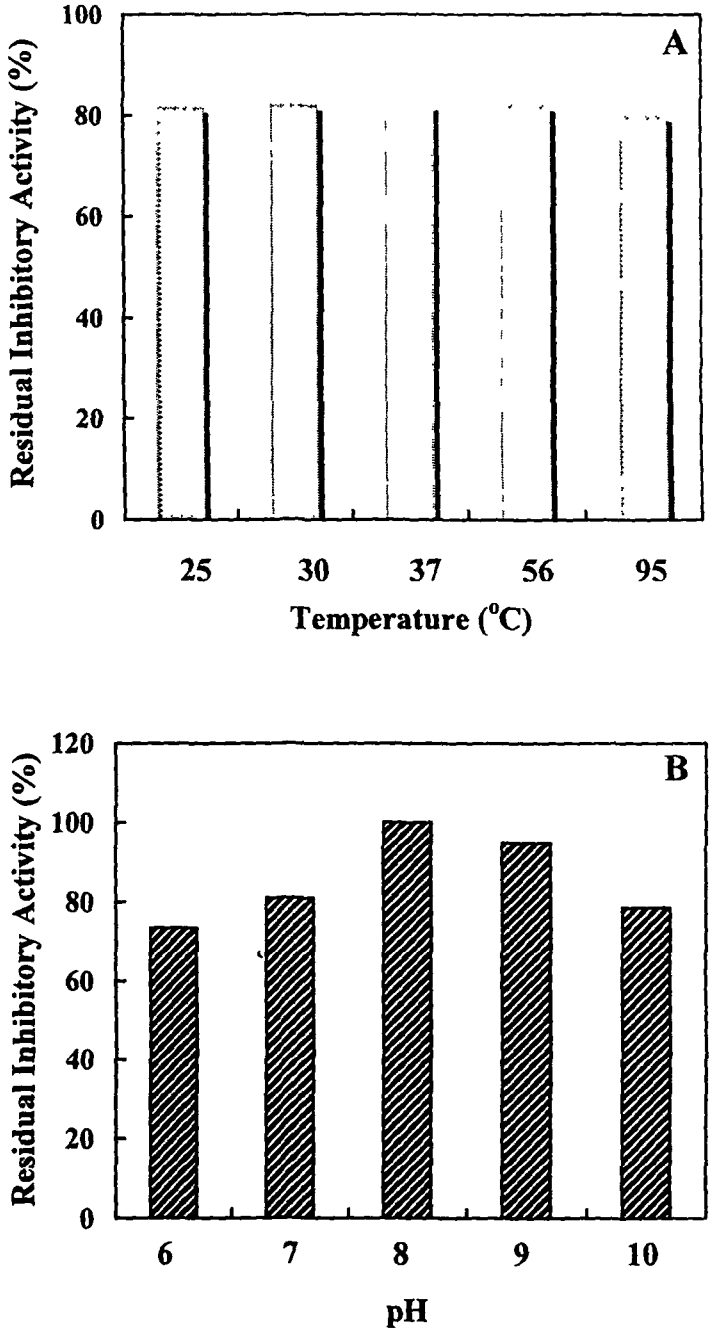


Fig.10.(A) Effect of temperature on inhibitory activity of *C. cajan* PI. Percent residual inhibitory activity after pretreatment at respective temperatures for 30 min.

(B) Effect of pH. Percent residual inhibitory activity of *C. cajan* PI in the pH range 6-10.

Table VI

Effect of 2mM DTT treatment on the inhibitory activity of *C. cajan* PI

	Inhibitory Activity (%)
Untreated PI	85.00 ± 0.49
PI + DTT (30 min.)	50.00 ± 5.66
PI + DTT (60 min.)	18.00 ± 2.83

3.1.6 Fluorescence quenching studies

Comparative studies on the native and 2mM DTT- treated (reduced) *C. cajan* PI were performed by fluorescence emission spectroscopy. Fluorescence quenching data with two quenchers; acrylamide and potassium iodide were analysed according to equations (2) and (3). The Stern-Volmer plot and modified Stern-Volmer plot for native PI, reduced PI and NATA with acrylamide and KI are depicted in figures 11 and 12 respectively. K_{sv} and f_a values obtained for both samples are summarized in Table VII. Fig. 10 shows the quenching results obtained with acrylamide. Acrylamide which is a neutral and efficient quencher, quenches the fluorescence of tryptophan residues both exposed to the solvent and buried inside the protein. The Stern-Volmer plot for native *C. cajan* PI is linear i.e. all tryptophans in the PI are accessible to acrylamide. K_{sv} for reduced PI is greater ($1.3 \times 10^{-2} mM^{-1}$) as compared to native PI (1.0×10^{-2} mM^{-1}) indicating that tryptophan residues in the reduced PI are more exposed to the solvent.

In order to understand more about the environment of tryptophan residues in *C. cajan* PI, we performed quenching studies with the anionic quencher, iodide ion (Fig. 12). According to many studies using ionic quenchers like an iodide ion, it is well-known that the emission of exposed tryptophan residues are quenched selectively [240]. Iodide ion, being negatively charged and hydrated, is likely to quench only surface tryptophanyl residues, and its behaviour should also depend on the neighbouring charged groups [241]. Contrary to acryamide, KI quenching revealed a lesser degree of quenching in reduced PI as compared to native PI. This suggests that some tryptophans in the *C. cajan* PI are present in the vicinity of positively charged groups; hence inaccessible to KI quenching. The percentage of the total fluorescence quenched by the ionic quencher calculated from the modified Stern-Volmer plot using Eq. 3 was found to be ~50%. This suggests that approximately half of the total number of tryptophans present in PI are exposed to solvent. NATA was used as a reference for free tryptophan and its quenching behaviour was similar in both cases. To rule out the effect of ionic strength we measured the fluorescence spectra for PI with varying [NaCl] in the range of 3.3 - 33 mM (same as that of KI). No significant change was observed on the intensity of the steady-state fluorescence spectra

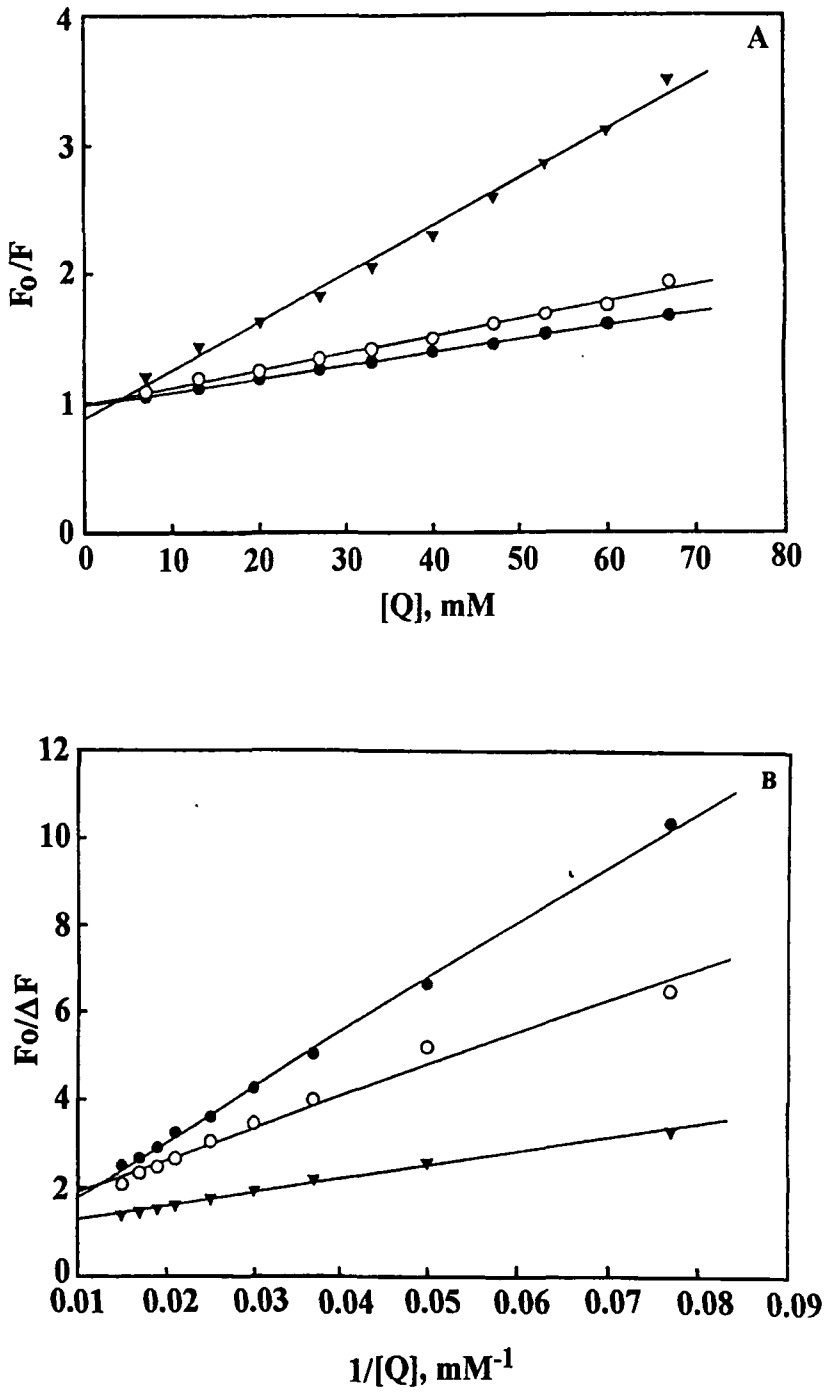
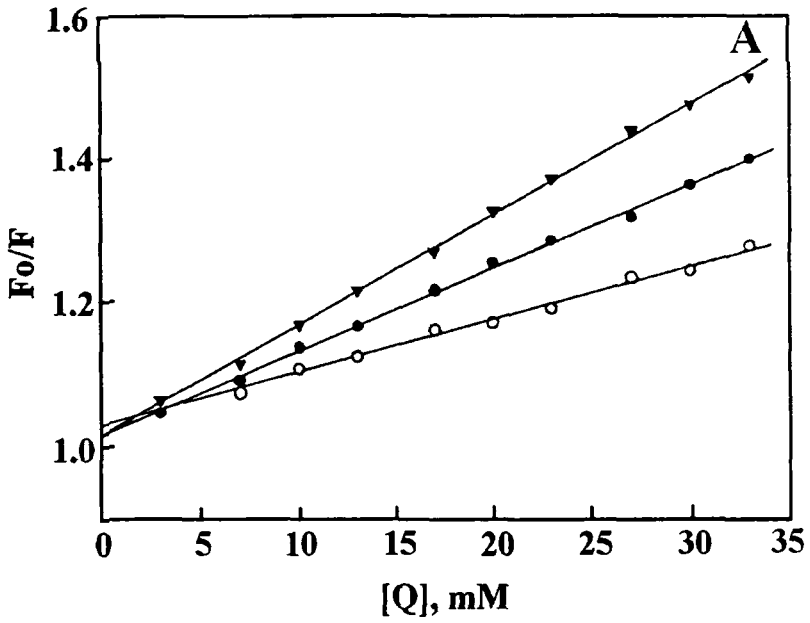


Fig.11. Acrylamide quenching of tryptophan fluorescence for native C. cajan PI (—●—), reduced PI (—O—) and NATA (—▼—) (A) Stern-Volmer plot (B) modified Stern-Volmer plot.



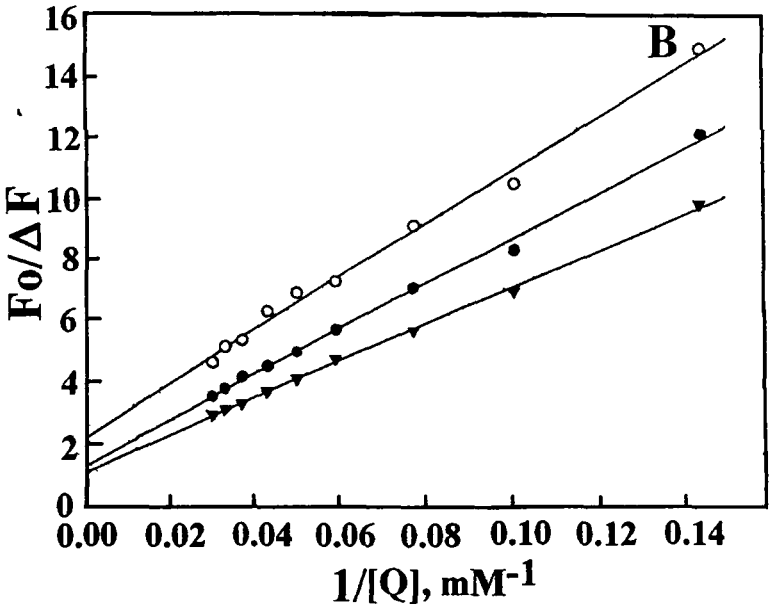


Fig.12. KI quenching of tryptophan fluorescence for native *C. cajan* PI (—•—), reduced PI (—O—) and NATA (—▼—) (A) Stern-Volmer plot (B) modified Stern-Volmer plot.

Table VII

Fluorescence parameters for quenching of NATA, native *C. cajan* PI and reduced PI by Acrylamide and KI from equations (2) and (3)

Protein	K_{SV} (mM^{-1})		f_a	
	Acrylamide	**KI**	**Acrylamide**	**KI**
NATA	0.037	0.015	0.988	0.926
PI (native)	0.010	0.012	0.507	0.787
PI (reduced)	0.013	0.007	1.178	0.455

3.1.7 CD Conformational studies

The near-UV CD spectrum shown in figure13A for *C. cajan* PI at pH 7.0 exhibits a minima at 279nm which could be due to tyrosine residues as previously reported for horsegram proteinase inhibitor by Ramasarma and co-workers [242]. A maximum at 250 nm is also observed which is attributed to disulfide linkages in the molecule [243]. In the far-UV CD spectrum (Fig. 13B) of *C. cajan* PI at pH 7.0, a strong negative band centered at 200 nm and a positive band at 190 nm are observed. This is indicative of unordered structure with disulfide bonds as reported previously for horse gram PI [242]. The class of unordered proteins includes many oligopeptides, short polypeptides with disulfide bonds or prosthetic groups, and denatured proteins. These oligo- and polypeptides usually show a CD spectrum with a strong negative band near 200nm, and some weak bands between 220 and 230 nm which can have either positive or negative signs [244]. Most of these inhibitory polypeptides show random coil secondary structure [245].

A probe into the structural features of *C. cajan* PI at three different pH values, viz, 7.0, 2.0 and 10.0 yielded a largely stable conformation (Figs. 13A and B). Near-UV CD spectra show minor conformational alterations at the pH values examined. In the far-UV CD spectrum observed at pH 2.0, *C. cajan* PI retains the observed CD bands at 200 nm and 190 nm; the depth of the negative band at 200nm being slightly larger than that for pH 7.0. However, the spectrum is altered at alkaline pH. The positive band at 190 nm is lost and the 200 nm band is red-shifted. This suggests that the *C. cajan* PI is a relatively stable protein as its conformation is only slightly affected at low pH.

Figure 14 depicts the effect of DTT on the *C. cajan* proteinase inhibitor. In the presence of 1mM and 10 mM DTT, the PI clearly shows a loss in the 257nm positive band which has been correlated to loss of disulfide linkages [242]. The trough at 279nm is abolished, and new minima appeared at 278nm and 284nm in the presence of 1mM DTT indicating gross conformational alterations in the overall tertiary structure. Far-UV CD spectra also show pronounced conformational changes in the reduced PI samples. The positive band at 190nm attributed to disulfides is lost in the presence of 1mM DTT. The strong negative band near 200 nm observed in native PI is greatly altered in the reduced preparations.

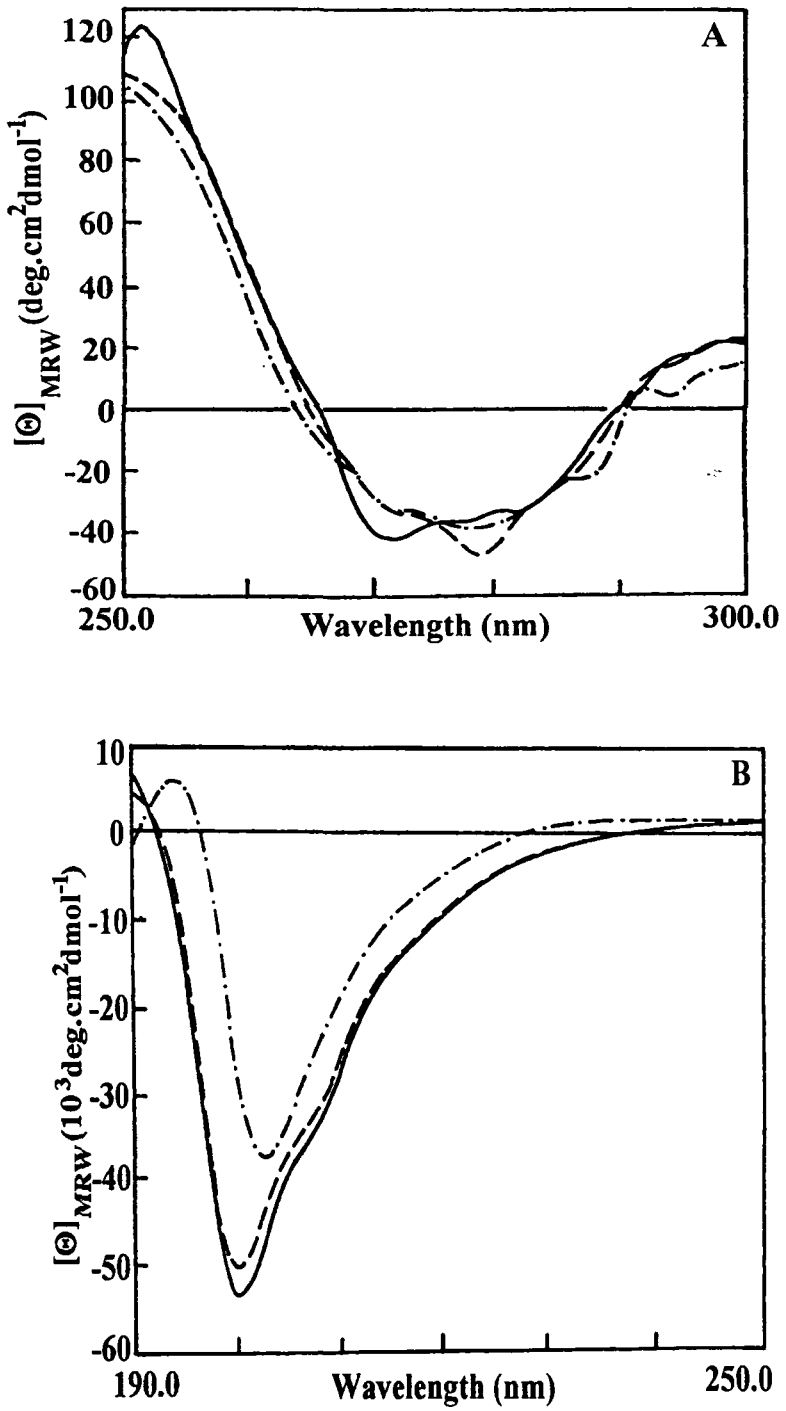


Fig.13. Effect of pH on the conformation of *C. cajan* PI. **(A)** Near-UV and **(B)** Far-UV CD spectra of the PI at pH 7.0 (-----), pH 2.0 (——) and pH 10.0 (—•—).

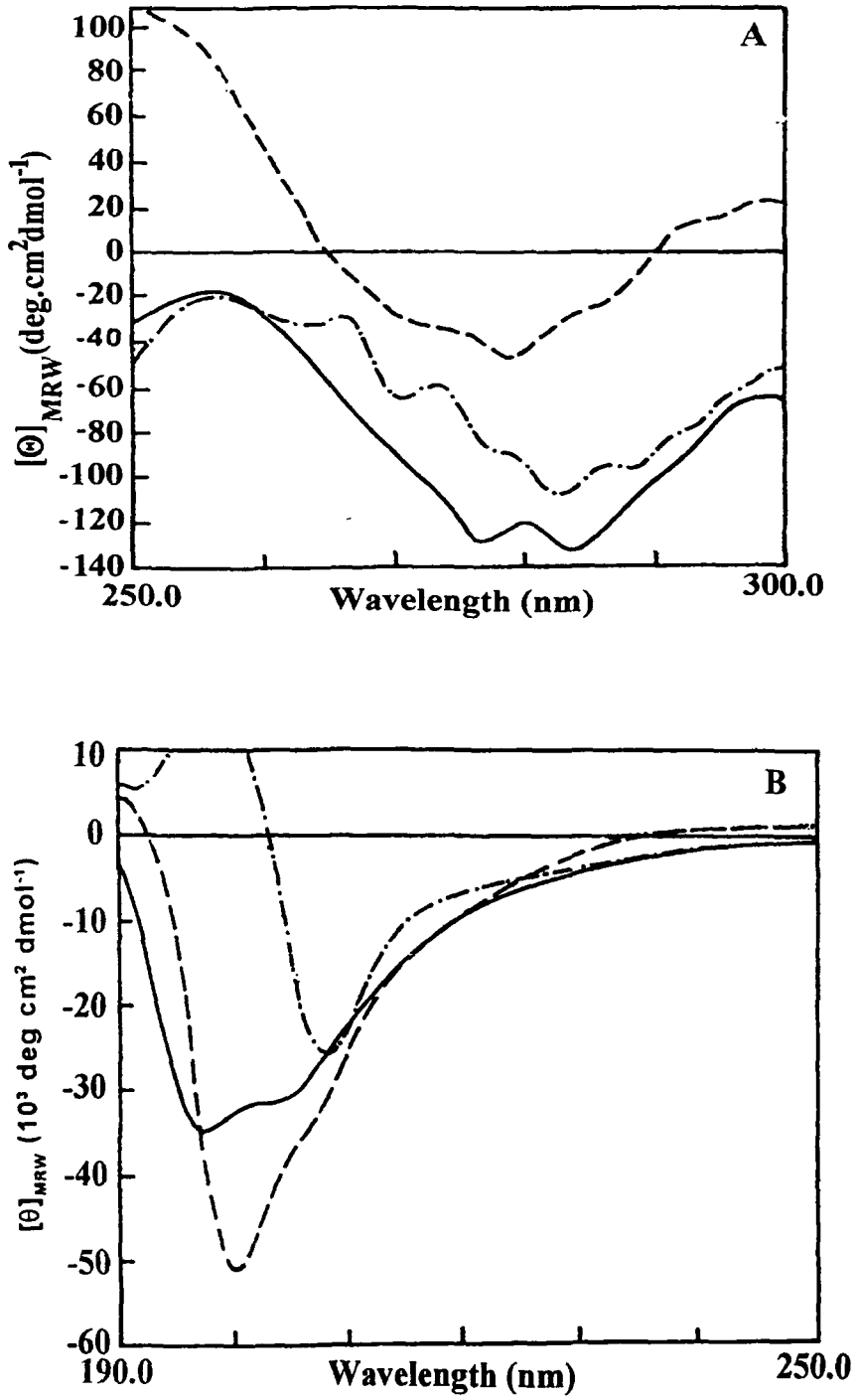


Fig.14. Circular Dichroism: (A) Near-UV and (B) Far-UV CD spectra of native *C. cajan* PI (-----), PI + 1mM DTT (——) and PI+10mM DTT (—•—).

The above-discussed conformational alterations in the *C. cajan* PI as studied by CD spectroscopy undoubtedly suggest the role of disulfide linkages in the structure and function (as observed by decrease in inhibitory activity upon reduction) of the proteinase inhibitor.

3.1.8 Inhibition kinetics

The kinetics of association of *C. cajan* PI to trypsin and α-chymotrypsin were analysed by measurements of the loss of enzyme activity in the presence of specific chromogenic substrates. All analyses showed a linear dependence of $K_{obs,}$ on inhibitor concentration within the concentration range covered, the slopes of these plots giving k_{ass} (Table VIII). The PI binds to trypsin with about 200-fold higher k_{ass} than to α-chymotrypsin. The dissociation equilibrium constant, K_d, for the binding of *C. cajan* PI to trypsin and α-chymotrypsin was determined as K_i by measurements of the decrease caused by the inhibitor of the equilibrium rate of cleavage of a chromogenic substrate by the respective proteinase (Table VIII). As can be seen from the table, the affinity of *C. cajan* PI for trypsin is much higher to that for α-chymotrypsin. The main reason for the high affinity for trypsin is rapid association (k_{ass} ~10^6 M^{-1} s^{-1}) whereas the rate of binding to α-chymotrypsin is much slower.

3.1.9 N-terminal sequence

The N-terminal protein sequence of *C. cajan* PI is given in Table IX. A comparison with other known inhibitors to check sequence homology revealed that it resembles Kunitz inhibitors especially Soybean trypsin inhibitor (Kunitz). Conserved residues (eg. Asp5) typical for the members of the Kunitz soybean inhibitor superfamily, can be found in the sequences of all these proteins. All plant protein proteinase inhibitors can be divided into at least ten structural groups (families) on the basis of their primary structure, number of disulfide bonds and their localization, and the disposition of their reaction centers [246]. Protein inhibitors of proteinases from the Kunitz soybean trypsin inhibitor (STI) superfamily, previously found mainly in legumes and cereals, form one of these groups with molecular weights around 20-24 kD and two disulfide bonds.

Table VIII

Kinetic parameters for the interaction of *C. cajan* PI with trypsin and α-chymotrypsin

Enzyme	$k_{ass}(M^{-1}s^{-1})$	K_i (M)
Trypsin	$(1.0 \pm 0.2) \times 10^{6}$(3)[c]	$(2.8 \pm 0.1) \times 10^{-10}$(3)[c]
α-Chymotrypsin	$(5.0 \pm 0.2) \times 10^{3}$(3)[c]	$(4.0 \pm 0.3) \times 10^{-5}$ (3)[c]

[c]Numbers in parantheses denote the number of times the experiments were repeated.

Table IX

The N-terminal sequence of *C. cajan* PI aligned with other known Kunitz type trypsin inhibitors

Inhibitor (Source)	Initial position	Sequence	Reference/ NCBI_Tax ID
Erythrina variegata trypsin inhibitors			
ETIa	1	V **L** L D G	[247]
ETIb	1	E **L** **V** D V	[247]
Putative Kunitz-type tuber invertase inhibitor (Fragment) *Solanum tuberosum*	3	F **L** **V** **L** S	4113
***Cajanus cajan* proteinase inhibitor**	**1**	**G** **L** **V** **L** **D**	
Soybean (*Glycine max*) Trypsin Inhibitor B (Kunitz)	1	D F **V** **L** **D**	3847
Kunitz inhibitor from *Peltophorum*	1	D F **V** **L** **D**	[248]
Dimorphandra mollis trypsin inhibitor-II(DMTI-II)	1	**L** **V** Y **D**	[249]
Dimorphandra mollis trypsin inhibitor(DMTI)	2	Q **V** F **D**	[250]
Kunitz-type serine protease inhibitor(BbKI) from *Bauhinia bauhinioides*	1	S V **V** V **D**	166014
α-chain of proteinase iso inhibitor DE5 from seeds of Carolina (*Adenanthera pavonina* L)	1	R E L **L** **D**	[251]

3.1.10 Thermal denaturation studies on *C. cajan* proteinase inhibitor using circular dichroism

Resistance towards loss in structure and/or function of a particular protein due to temperature-induced denaturation is a measure of its stability. No study on the stability and stabilization of protein macromolecules is complete without a measure of their tolerance towards thermal stress. Several proteins express varying degrees of stability when subjected to heat denaturation. Keeping in view the stability of *C. cajan* proteinase inhibitor towards thermal inactivation (Fig.10A), the effect of temperature on the structure of the protein inhibitor was probed using circular dichroism spectroscopy. The thermal transition curves for *C. cajan* PI at pH 7.0 and 2.0 obtained by plotting ellipticity ($[\Theta]_{MRW}$) values at 280 nm as a probe for tertiary conformation are depicted in figure15. The transition, as seen from the figure is a two-state (N↔D) reversible process at neutral pH. The mid-point of transition (T_m) was ~ 63°C for the inhibitor at pH 7.0. Increase in temperature causes the weakening of a number of bonds in a protein molecule. The first affected are the long-range interactions that are necessary for the presence of tertiary structure. As these bonds are first weakened and broken, the protein attains a more flexible structure that eventually unfolds with further increase in temperature. A similar reversible thermal unfolding behaviour is also exhibited by the soybean Kunitz inhibitor [252]. It is noteworthy here, that the retention of inhibitory activity by protein PI at neutral pH even after exposure to extreme temperatures can now be understood in terms of its complete structural reversibility. The transition at pH 2.0 also shows a simple N↔D process with a T_m ~ 47°C. Figure inset portrays the fraction of protein denatured with respect to temperature. It is quite clear from the figure that the protein proteinase inhibitor is more susceptible towards temperature-induced denaturation at acidic pH; as the T_m (pH2.0) < T_m (pH7.0). In other words, the temperature at which the PI is half-denatured is lower for the inhibitor at acidic pH. This is unlike previous reports on the major trypsin inhibitor from the winged bean seed (*Phosphocarpus tetragonolobus* (L.) DC) that is reportedly stable to heat upto 60°C at neutral pH and exhibits enhanced stabilty towards higher temperatures at low pH [253].

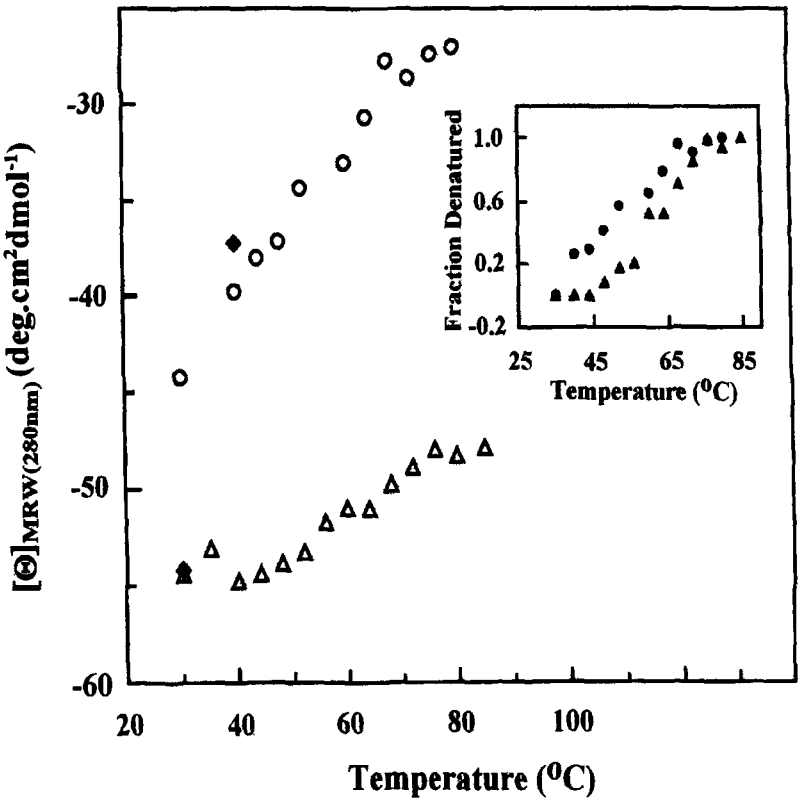


Fig.15. Thermal transition of *C. cajan* PI at neutral (Δ, ▲) and acidic (O, ●) pH. $[\Theta]_{MRW}$ at 280 nm is plotted against temperature. Figure Inset: Fraction of protein denatured as a function of temperature. The symbol (◆) represents the reversibility point after cooling.

Figure16A depicts the near-UV circular dichroism spectra of the inhibitor at different temperatures overlaid for comparison of changes in spectral features. As seen in the figure, the PI at pH 7.0, 35°C shows characteristic minima at 275nm and 279nm as also reported previously [8]. Alterations in ellipticities within the near-ultraviolet region (300-250nm) reflect modifications in the asymmetrical environment of aromatic residues. At 85°C, a decrease in the magnitude of all the negative CD bands is observed. The positive band around 250 nm is lost. This suggests that the overall tertiary conformation of the protein PI is getting altered. The spectrum recorded at 35°C; after cooling from 80°C exhibits a similar spectral pattern as the spectra recorded at 35°C confirming the complete reversibility of the change observed. The temperature induced alterations at 80°C for *C. cajan* PI at acidic pH are more prominent as evidenced from figure16B. A significant decrease in the ellipticities of the minima at ~276 nm and ~282 nm is observed at this temperature. The positive ellipticity at ~250 nm is also decreased. Exposure to low pH causes protonation of the acidic amino-acid residues; resulting in an imbalance of the net charge on the native protein. The consequent intramolecular charge-charge repulsion may be one factor resulting in at least partial unfolding of the protein. Protonation of residues in close vicinity of the aromatic amino-acids may be the other reason that alters the asymmetric environment of the chromophoric groups and hence the altered CD signal. Upon rapid cooling to 35°C, the negative band intensities are increased as the protein attempts to regain its original conformation. However, complete reversal is not achieved.

Figure 17A shows the comparative far-UV CD spectra of the *C. cajan* PI (neutral pH) at different temperatures. As seen in the figure, the proteinase inhibitor shows a single minimum around 204 nm at 35°C. At elevated temperatures (85°C), the intensity of this band is reduced and red-shifted to approximately 208nm. Furthermore, there is an appearance of shoulder-like regions around 214nm and 222nm. A CD spectrum with a strong negative band near 200nm, and some weak bands between 220 and 230 nm which can have either positive or negative signs is associated with oligo- and polypeptides belonging to the unordered class of proteins [244]. Upon cooling, there is a significant reversal of spectral features, viz. restoration of minima at ~200nm and the positive CD band around 190nm.

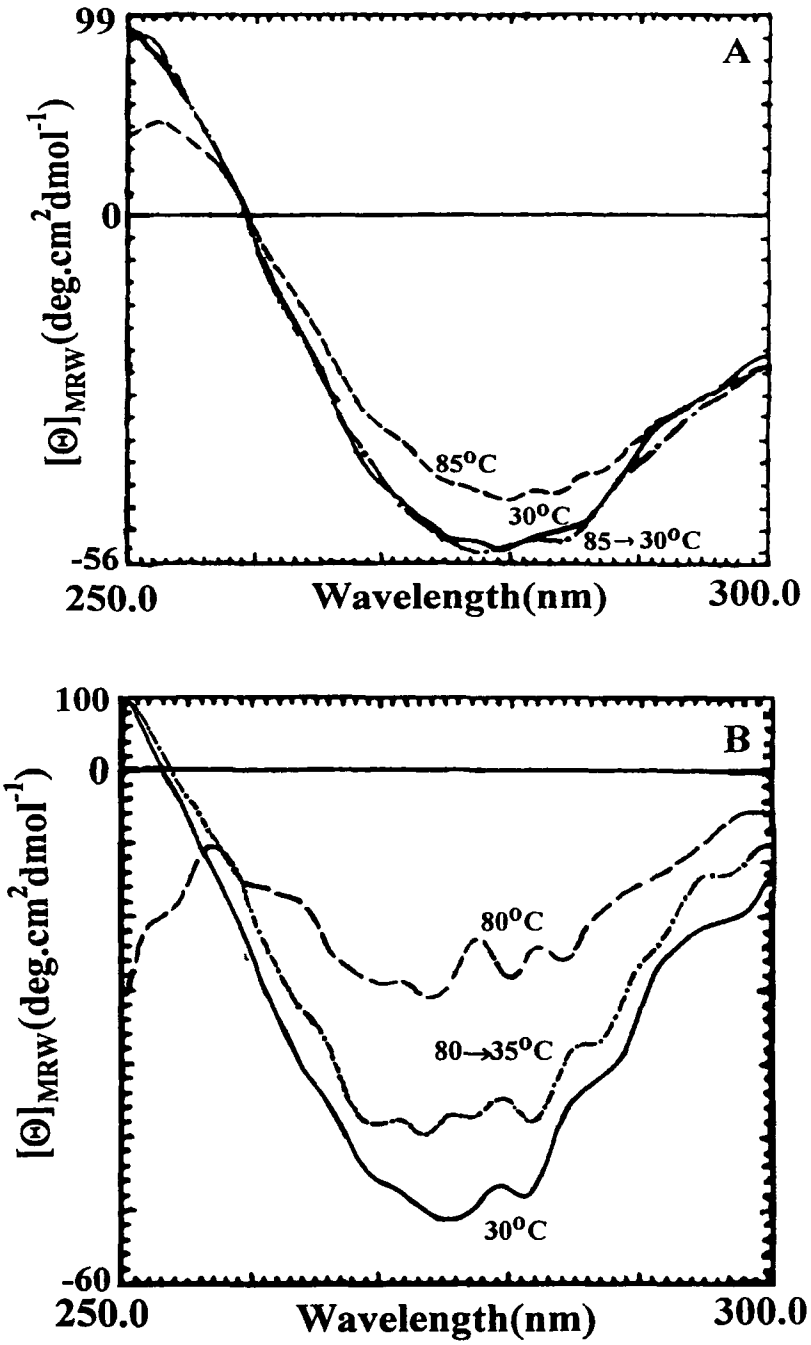


Fig.16. Effect of temperature on the tertiary conformation of *C. cajan* PI. Near-UV CD spectra of the PI at pH 7.0 (**Panel A**) and pH 2.0 (**Panel B**). Curve (——) represents the protein at 35°C, curve (- - - -) represents the protein at 85°C and curve (—•—) is the spectrum of protein inhibitor rapidly cooled down to 30°C

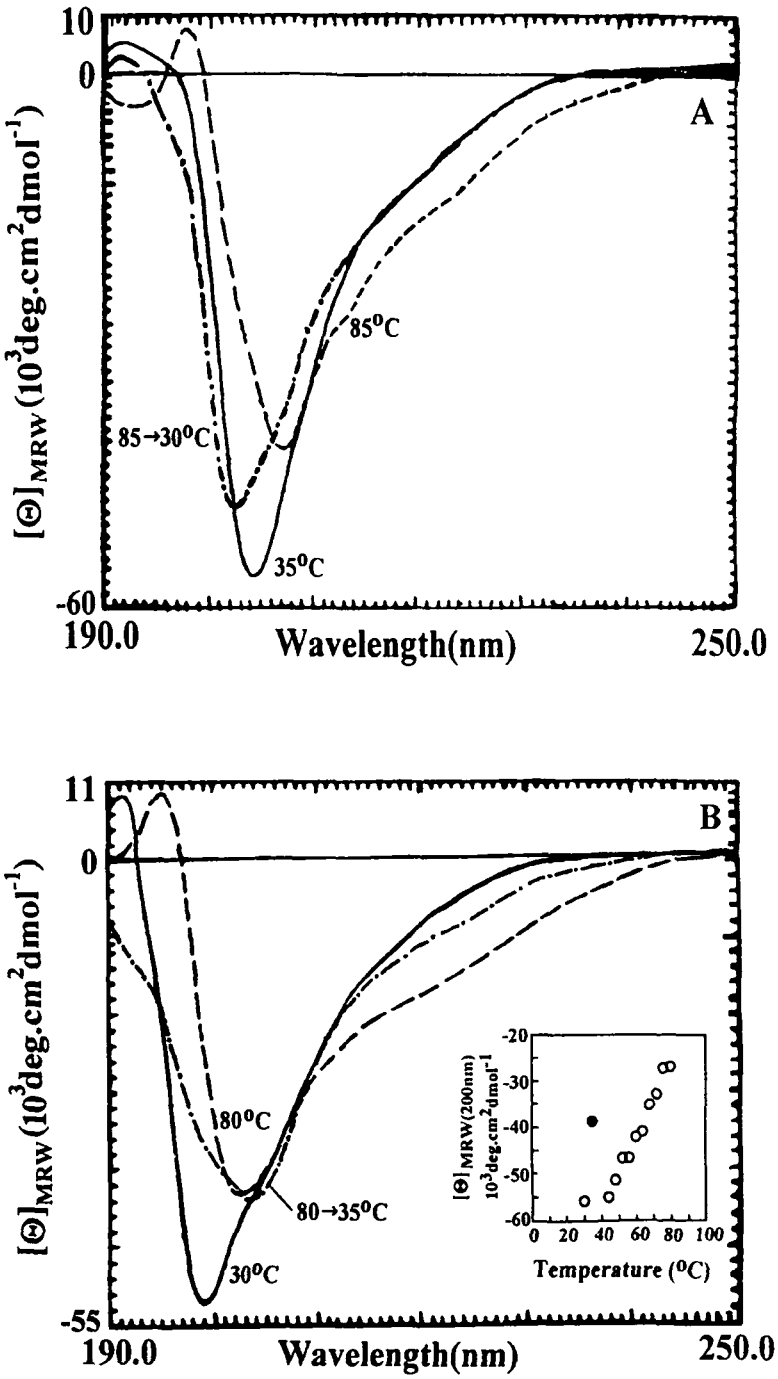


Fig.17. Effect of temperature on the conformation of *C. cajan* PI. Far-UV CD spectra of the PI at pH 7.0 (**Panel A**) and pH 2.0 (**Panel B**). Curve (——) represents the protein at 30°C, curve (— —) represents the protein at 80 or 85°C and curve (— • —) is the spectrum of protein inhibitor rapidly cooled down to 30 or 35°C. **Figure17B inset**: Thermal transition of *C. cajan* PI at pH 2.0 (O, ●); [Θ] MRW at 200 nm is plotted against temperature.

Fig.17B shows the spectra of the PI at acidic pH in the peptide absorption region under similar conditions. The proteinaceous inhibitor under acidic conditions exhibits spectral features almost indistinguishable from the native PI at pH 7.0. When exposed to high temperature (80°C), the negative extremum at ~200 nm is decreased and red-shifted by 3-4 nm accompanied by the appearance of a shoulder around 220nm. However, upon cooling, no reversal in the spectral features is observed. This indicates that the protein is more susceptible towards temperature-induced denaturation at acidic pH that can be attributed to the disruption of stabilizing electrostatic interactions. Increased number of salt bridges, often organized in networks, have been recognized to play a predominant role in the stabilization of proteins from higher thermophiles [254,255]. Figure inset shows the transition curves of the *C. cajan* PI obtained as change in ellipticity at 200 nm versus temperature at pH 2.0. As stated earlier, the trypsin/chymotrypsin inhibitory polypeptide from *C. cajan* displays a far-UV CD spectrum with a single negative maximum at around 200 nm and a positive band near 195 nm, a pattern consistent with a polypeptide having an unordered secondary structure [242]. Hence, this wavelength was chosen to monitor the changes in backbone secondary structure at low pH as a function of temperature.

The transition at pH 2.0 was observed to follow an N↔D transition with a mid-point around 60°C. Comparing this to the transition in figure 1, we infer that changes in secondary structure of the protein at low pH begin only after almost half of the tertiary structure has been lost. As with the near-UV CD conformation, the far–UV CD ellipticity is also reversed only partly (~50%). A plot of fraction of protein denatured (f_D) versus temperature for near- and far- ultraviolet changes in the PI at pH 2.0 (Fig.18) clearly shows that consequent to heat-induced stress, tertiary structure of the PI is the first to undergo conformational changes followed by the secondary structure.

Trends commonly associated with elevated thermostability in proteins include relatively small solvent-exposed surface area, increased packing density that reduces cavities in the hydrophobic core, optimization of hydrophobic interactions, decreased length of surface loops, and hydrogen bonds between polar residues [256,257-261]. Whereas thermostability likely results from optimizations of all these mechanisms, the

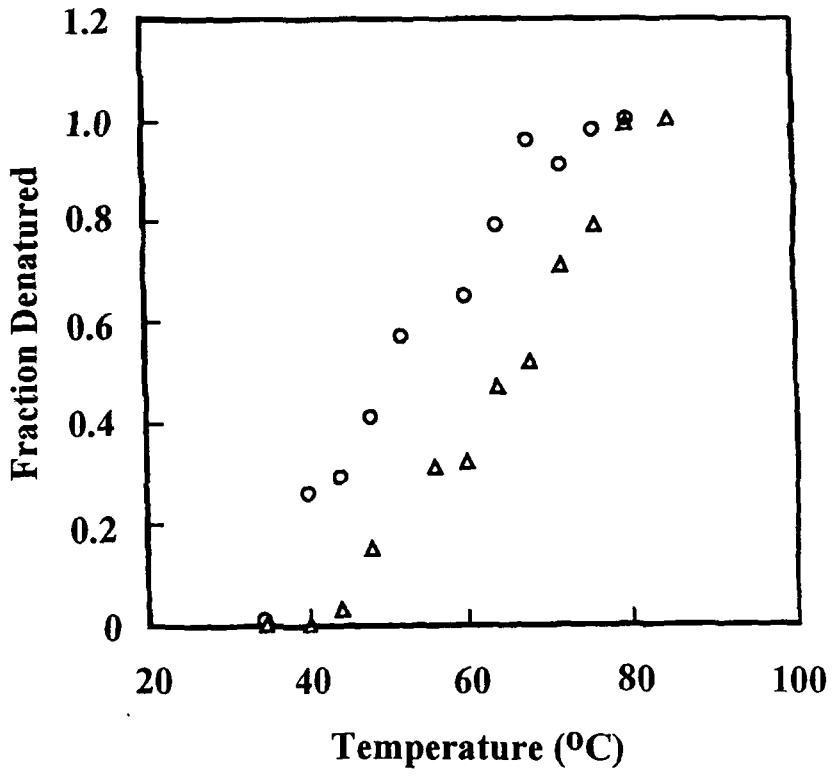


Fig.18. Fraction of protein denatured as a function of temperature for *C. cajan* PI at pH 2.0. Near-UV (O) and far-UV (Δ) changes calculated from $[\Theta]_{MRW}$ measurements at 280nm and 200nm respectively are plotted against temperature.

presence of enriched polar interactions has been a common theme among thermophilic proteins. A critical role for ion-pairs in stabilization has been inferred from the recently solved structures of several proteins from hyperthermophiles [262].

Other factors affecting protein stability are conformational flexibility [12], glycosylation [13], protein-protein interactions [14] and the presence of disulfide bonds [15]. Disulfide bonds are believed to increase the stability of the native state of a protein by decreasing the conformational entropy of the unfolded state due to the conformational constraints imposed by the cross-links (i.e. decreasing the free energy of the unfolded state). Protein proteinase inhibitors from plants, generally implicated in plant defense are a widely studied group of proteins often rich in disulfide linkages. Following the quantification of disulfides in the *C. cajan* PI, and the proposed role of disulfide linkages in conferring protein stability, it was thought relevant to study the effect of a reducing agent on protein stabilization/destabilization under conditions of thermal stress.

Figure 19A depicts the temperature-induced transition in $[\Theta]_{MRW}$ values at 257nm at both the pH values in the presence of 10mM dithiothreitol. A maximum at 257nm or 250nm is generally attributed to disulfide linkages in the protein molecule [242,243]. Hence, this wavelength was chosen in order to assess changes in tertiary structure following exposure to a reducing agent as a function of temperature. It can be seen in the figure that ellipticity at 257nm decreases continuously with temperature and is at a minimum around 70°C and above. This indicates gross alterations in the bridging disulfides leading to decreased absorption in this region that are irreversible. For the PI at pH 2.0, the $[\Theta]_{MRW}$ value at 257nm is already decreased; however, changes as a result of increased temperature are less drastic and are easily reversed.

Near-UV CD studies at pH 7.0 under reducing conditions show that addition of dithiothreitol affects the tertiary conformation of the PI to a great degree owing to the reduction of disulfide linkages (figure not shown). Treatment of the inhibitor to dithiothreitol also led to a significant reduction in its inhibitory potential [8] thereby implying the importance of disulfide bonds in maintaining the three-dimensional conformation essential for its function. Studies on the *Cucurbita maxima* trypsin inhibitor-V with an engineered disulfide bond revealed that an enthalpy-entropy

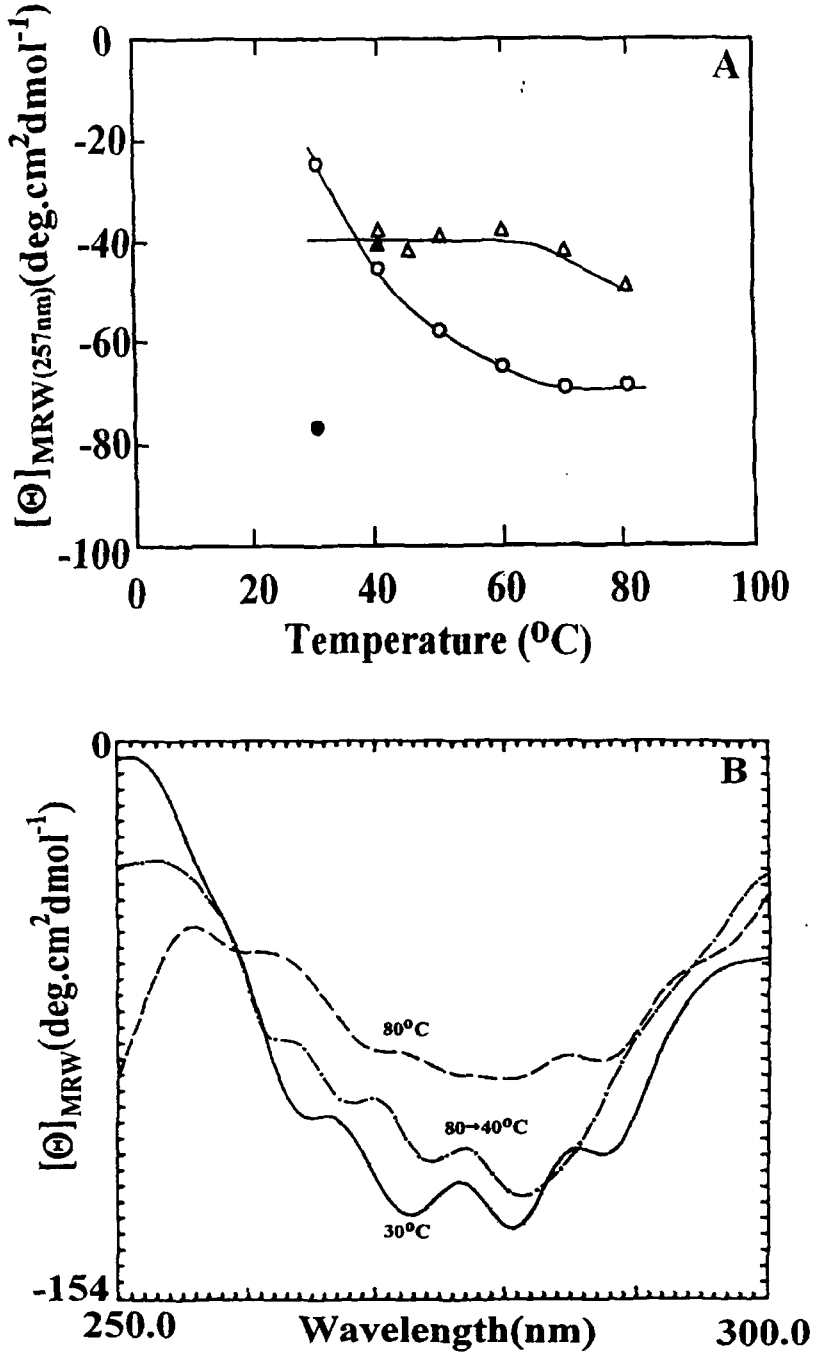


Fig.19. **(A)** Thermal transition of *C. cajan* PI at neutral (O) and acidic (Δ) pH in the presence of 10mM dithiothreitol. $[\Theta]_{MRW}$ at 257nm is plotted against temperature. Symbols (●,▲) represent the reversibility points after cooling.
(B) Near-UV CD spectra of the reduced PI at pH 2.0. Curve (——) represents the protein at 30°C, curve (— —) represents the protein at 80°C and curve (— • —) is the spectrum of protein inhibitor rapidly cooled down to 40°C.

compensation accompanies disulfide bond effects and protein stabilization is profoundly altered by hydrophobicity of both native and denatured states, altered flexibility near the cross-link and residual structure in the denatured states [263]. Figure 19B exhibits the comparative near-UV CD spectra at pH 2.0 under similar conditions. The characteristic spectral features seen at 35°C are altered at 80°C with respect to their intensities. On rapid cooling to 40°C, the intensities of negative CD ellipticities are restored to some extent.

Figure 20A depicts the comparative far-UV CD spectra of the *C. cajan* PI (neutral pH; PI + dithiothreitol) obtained at 40°C, 80°C and after cooling back from 80°C to 40°C. The minimum is now observed at ~210nm alongwith a shoulder above 220nm. It is noteworthy that at 80°C, the intensity of this band is decreased accompanied by a concomitant enhancement in the intensity and a broadening of the shoulder around 220nm. When the protein is cooled back to 40°C, a partial reversal in the features is observed. Figure 20B shows the far-UV CD spectra of the PI at pH 2.0 under reducing conditions at 30°C, 80°C and the spectrum obtained after cooling back to 40°C. It is interesting to note that the PI at low pH in the presence of dithiothreitol shows a single negative trough at 210nm. The intensity of this band is surprisingly increased with increase in temperature alongwith the appearance of another negative trough near 222nm. The spectrum is reminiscent of an alpha-helix type of secondary structure. Increase in negative ellipticities (217nm and 207nm) with increase in temperature have been reported earlier in case of αA-and αB-crystallin homoaggregates [264]. Lowering the temperature back to 40°C causes the disappearance of the minimum near 222nm as the protein conformation reverts to its original state.

Irreversible thermal denaturation of many proteins is generally attributed to alterations such as aggregation, autolysis or chemical alteration of residues [265] that lock the protein in a final state that is unable to fold back to the native structure. On the other hand, thermal reversibility is a phenomenon that excludes all the above-mentioned effects. Taken together, the results obtained with heat denaturation of *C. cajan* proteinase inhibitor suggest that such inhibitory polypeptides containing disulfide bonds and an unordered conformation are largely stable towards heat denaturation; retaining their inhibitory activities at extremes of temperatures. However; at low pH, this unusual

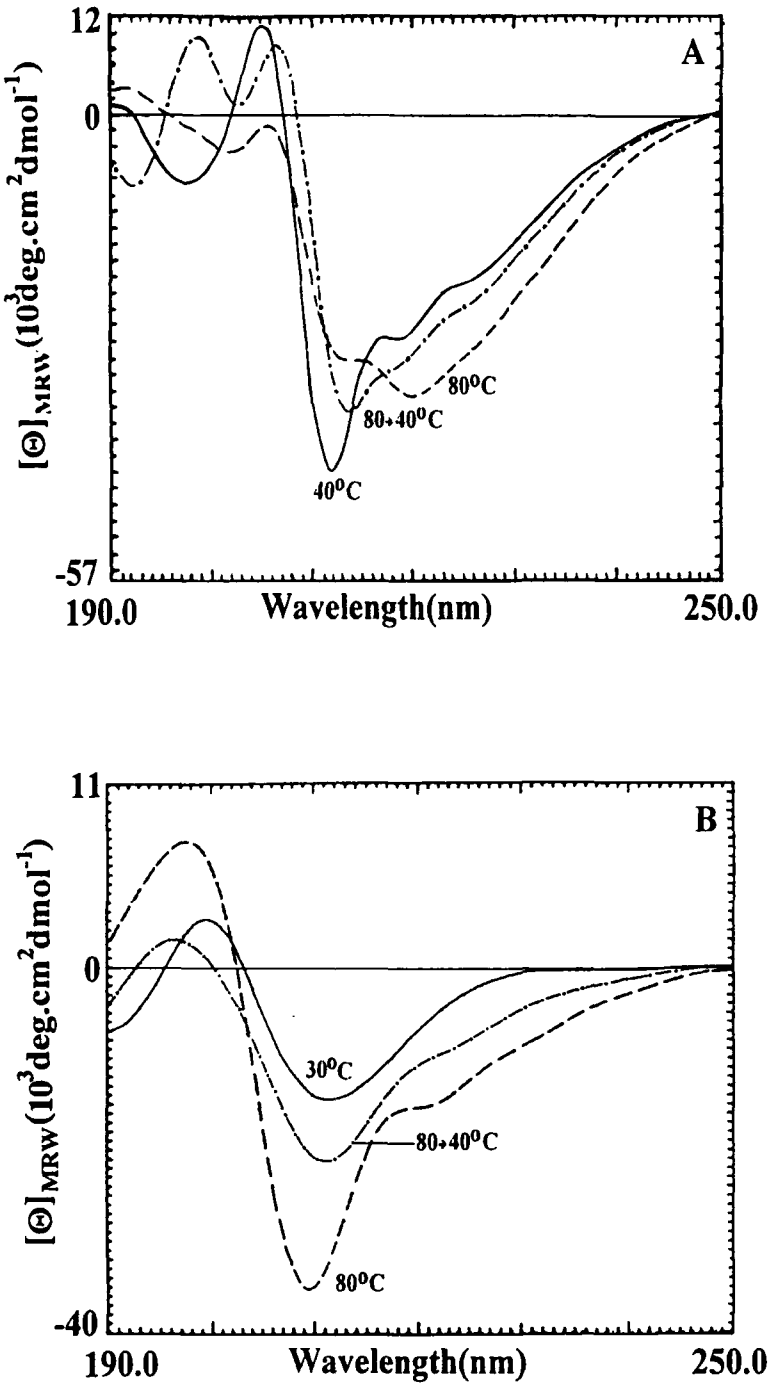


Fig.20. Effect of temperature on the secondary structure of *C. cajan* PI at neutral and acidic pH under reducing conditions. Far-UV CD spectra of the reduced PI at pH 7.0 (**Panel A**) and pH 2.0 (**Panel B**). Curve (——) represents the protein at 30 or 40°C, curve (— —) represents the protein at 80°C and curve (— • —) is the spectrum of protein inhibitor rapidly cooled down to 40°C.

Table-X

Mid-point of thermal transition (T_m) for the *C. cajan* proteinase inhibitor (PI) at neutral and acidic pH.

Region of transition (wavelength)	T_m (°C)	
	pH 7.0	pH 2.0
Near-UV CD (280nm)	63°C	47°C
Far-UV CD (200nm)	-----	60 °C

stability is reduced, probably due to loss of electrostatic interactions necessary in maintaining the structure. In the presence of dithiothreitol, a potent reducing agent of disulfides, the stability of the PI at neutral pH is more susceptible to heat denaturation as compared to that at low pH.

3.1.11 Effect of detergents

Keeping in view the wide applicability of detergents in membrane protein solubilization, electrophoresis, etc., it becomes imperative to develop a better understanding of the effects of detergents on proteins. Moreover, proteinase inhibitors and detergents are routinely used together in cell lysis buffers to inhibit unwanted proteolysis and facilitate membrane protein solubilization in protein purification procedures. Absence of characteristic features in the far-ultraviolet region (190-250 nm) representative of hydrogen bonded α-helical or β-structures in the Kunitz-type *C. cajan* (pigeonpea) inhibitor (as also soybean Kunitz inhibitor) suggest the role of hydrophobic interactions in inhibitor stability. Thus, a study of the conformational sensitivity of such inhibitors to the amphiphilic alkyl sulfates is of a considerable interest. Figure 21A shows the effect of increasing sodium dodecyl sulfate on the ellipticities ($[\Theta]_{MRW}$) of *C. cajan* PI at 208 nm and 222 nm. Slight increase in negative ellipticities at both the wavelengths is observed; however, this does not lead to the formation of ordered structure *per se*. The interaction of sodium dodecyl sulfate with proteins has been extensively studied by many groups, and their works are reviewed by Tanford [266] and Steinhardt and Reynolds [267]. SDS generally interacts with proteins such that dislocation of side chain chromophores occurs and helix formation is promoted [268]. Absence of any significant alteration in the near-UV CD spectrum of SDS-*C. cajan* PI complex (Fig.21B) suggests that the spectrum is dominated by the characteristics of the protein and the effect of detergent on protein conformation is negligible. The effect of different detergents (nonionic or ionic) on protein structure reflects the chemical nature of the detergents as well as the protein, the *latter* being applicable in this case.

Figure 22A represents the plot of $[\Theta]_{MRW}$ at 205 and 222 nm versus detergent (sodium deoxycholate, DOC) concentration. As seen in the figure, the ellipticity value at 205 nm remains more or less unchanged upto 2.5mM DOC concentration after which an

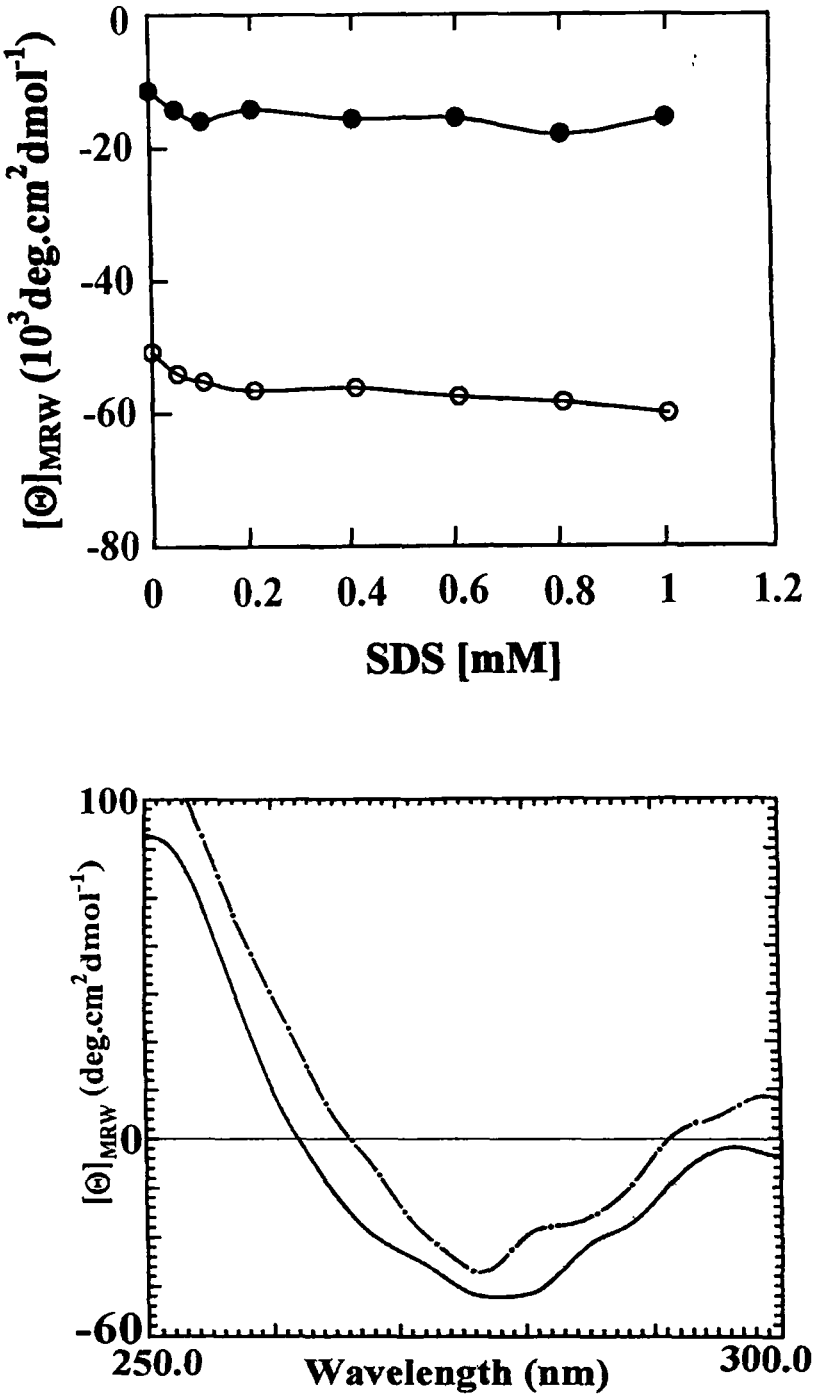
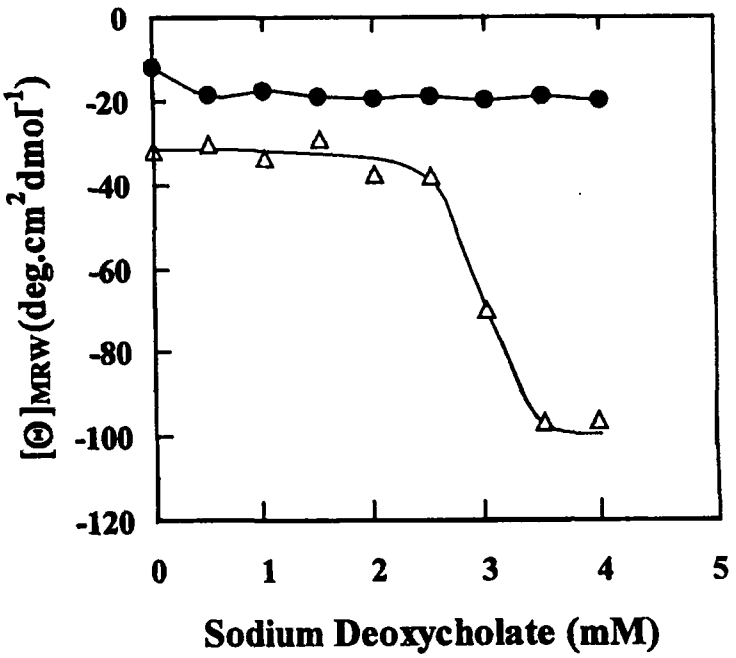


Fig.21. **(A)** $[\Theta]_{MRW}$ at 208 nm (O) and 222 nm (●) for *C. cajan* PI in the presence of increasing concentrations of sodium dodecyl sulphate.
(B) Near-UV CD spectrum of *C. cajan* PI in the absence (—•—) and presence (——) of 1mM SDS.



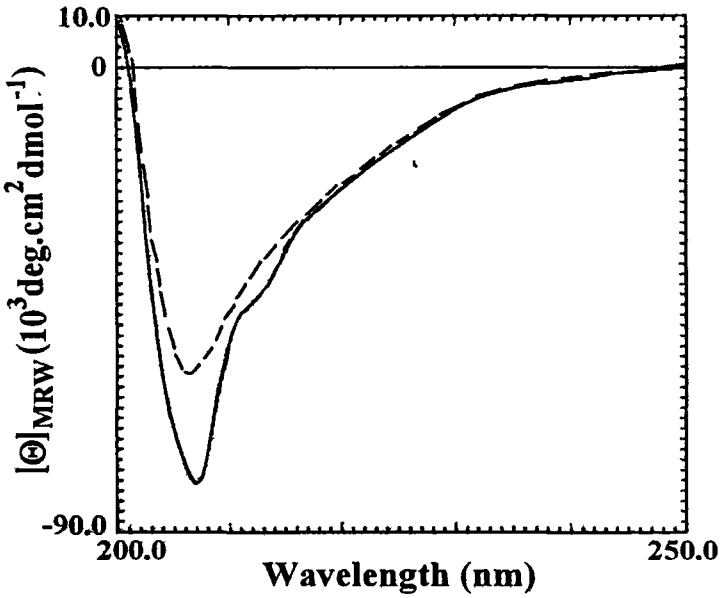


Fig.22. **(A)** $[\Theta]_{MRW}$ at 205 nm (Δ) and 222 nm (●) for *C. cajan* PI in the presence of increasing concentrations of sodium deoxycholate.
(B) Far-UV CD spectrum of *C. cajan* PI in the absence (— —) and presence (——) of 3mM DOC.

increase is observed. Since the MRE values at 222 nm remain largely unaffected, and spectrum in the presence of 3 mM DOC (Fig.22B) resembles that of *C. cajan* PI in the absence of detergent, (with a deeper trough at 205 nm), we infer that DOC stabilizes the unstructured protein without causing denaturation/alteration leading to helix formation.

Figure 23 shows the effect of varying concentration of CHAPS on the secondary structural conformation of *C. cajan* PI as monitored by change in $[\Theta]_{MRW}$ at 222 nm. A steady increase in MRE is observed beyond 5 mM CHAPS. The 205 nm band is decreased and red-shifted to ~210 nm and a slightly increased $[\Theta]_{MRW}$ is observed at around 220 nm.

The effect of detergents on protein function may be taken from activity measurements. Figure 24 shows the residual inhibitory activity of *C. cajan* PI in the presence of DOC and CHAPS. The ability of the proteinase inhibitor to inhibit the activity of trypsin is severely compromised in the presence of sodium deoxycholate whereas it is unaffected in the presence of zwitterionic detergent CHAPS. This is in agreement with the general belief that high concentrations of ionic detergents such as SDS/DOC cause loss of biological activity, while non-ionic detergents such as Triton X-100/ CHAPS show less deleterious effects. Since DOC causes minimal structural perturbations (Fig. 22A), the loss in inhibitory activity could be suggestively attributed to the binding of detergent molecules at or near its *reactive site*, causing loss of recognition of the PI as a substrate by the target protease. Such unusual/unpredicted effects on the activity have been reported earlier for a mutant protein (Bcl-X_L) in the presence of a non-ionic detergent [269].

Detergent-induced structural changes of proteins may be analyzed efficaciously by fluorescence emission as the fluorescence signal undergoes changes reflective of changes in protein conformation. Figure 25 depicts the relative fluorescence emission spectra of *C. cajan* PI in the 300-400 nm range in the absence and presence of 1mM sodium dodecyl sulfate (SDS) and 4mM sodium deoxycholate (DOC). Minimally enhanced fluorescence intensity in the presence of DOC (minor conformational change with ~4nm shifted λ_{max}) agrees well with circular dichroism data (see Fig.19) where no major change is perceived except for an increase in the negative CD band near 200 nm. An increase in the fluorescence emission intensity accompanied by a red-shifted λ_{max} of

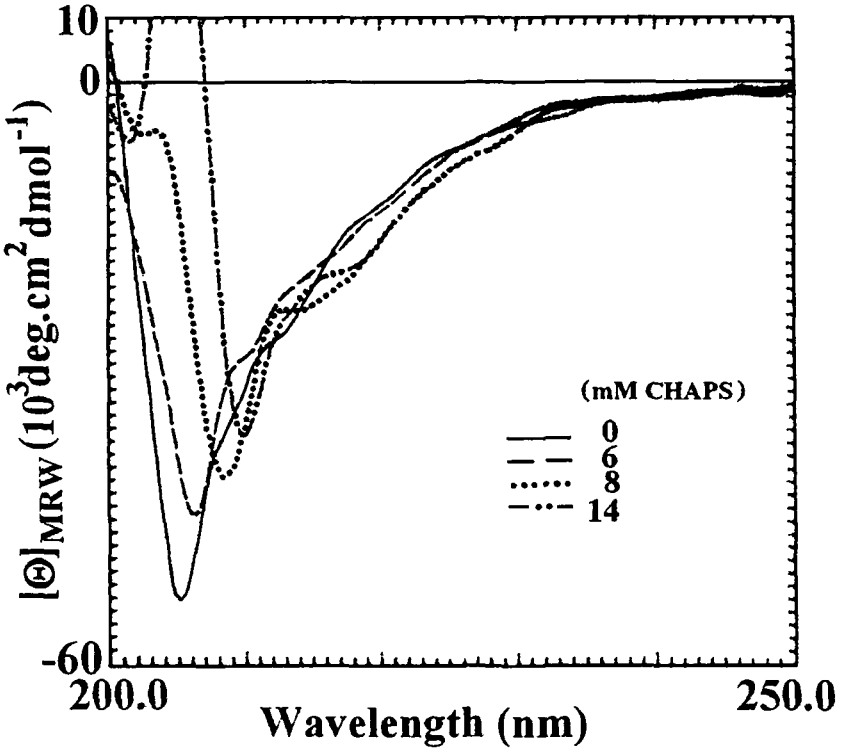


Fig.23. Far-UV CD spectra of *C. cajan* PI in the absence and presence of increasing concentration of CHAPS (0,6,8,14 mM).

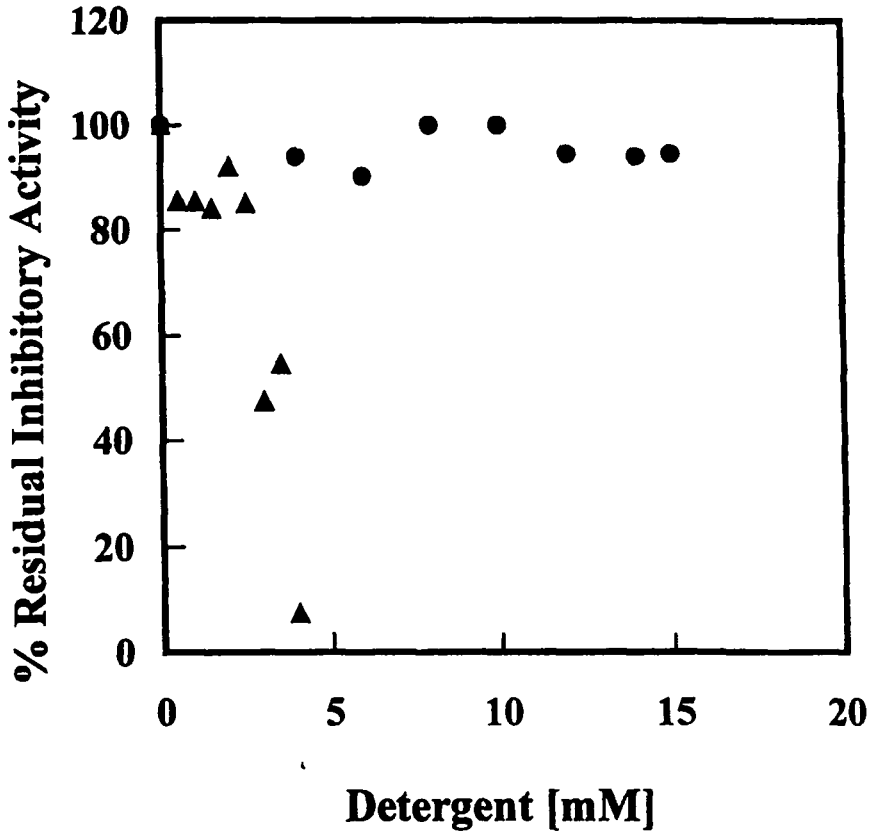


Fig.24. Percent residual inhibitory activity of *C. cajan* PI in the presence of CHAPS (●) and DOC (▲).

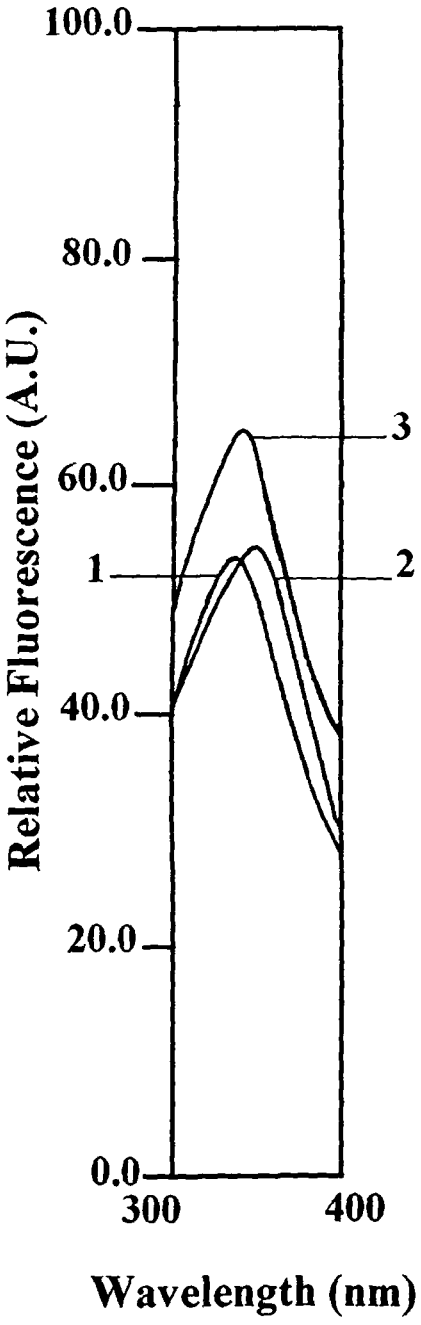


Fig.25. Relative fluorescence emission spectra of *C. cajan* PI in the absence (Curve 1) and presence of 1mM sodium dodecyl sulfate (SDS) (curve 2) and 4mM sodium deoxycholate (DOC) (curve 3).

~14nm in case of SDS suggests a more polar environment of aromatic amino-acids especially tryptophan in the presence of this detergent.

3.1.12 Effect of Hexafluoroisopropanol

Figure 26 depicts the effect of HFIP on the far-UV CD spectrum of *C. cajan* PI. As clearly seen from the figure, the fluorinated alcohol causes alterations in the secondary structural elements, leading to induction of ordered α-helical structure in an otherwise unordered polypeptide. Addition of HFIP to *C. cajan* PI induces a structural transition from the unstructured state to a predominantly helical conformation as suggested by intense, characteristic far-UV CD bands at 208nm and 222nm. A similar transition has been previously reported for melittin, a bee venom peptide that is unfolded in the absence of alcohol, but is transformed to α-helical structure upon addition of alcohols, fluoroalcohols or hexafluoroacetone hydrate (HFA) [270,271]. Induction of partial helix to an unstructured peptide is also observed for mouse prion protein peptide (residues 129-141) in the presence of trifluoroethanol [272]. Substituted fluoroalcohols are known to be strong inducers of helical secondary structure in proteins. Several reports on the induction of α-helix in all β-sheet proteins and increase in helix in helical proteins exist but reports on structure formation in unordered polypeptides are few. With respect to observed results on the unordered *C. cajan* PI in presence of HFIP, it is indeed interesting to note that although a polypeptide chain has the required amino-acids conferring propensity to form α helix; it does not exist in a folded structure. This property is manifested only under certain solvent conditions such as high concentrations of fluorinated alcohols (eg. hexafluoroisopropanol). Hirota *et al.* [273] initially suggested that cooperative formation of micelle-like clusters in case of HFIP was an important phenomenon. Hong *et al.* [270] examined the role of several factors *viz.*, dielectric constant, hydrogen bond and clustering of alcohol molecules. Their observations on bovine β -lactoglobulin A (a predominantly β -sheet protein) and melittin (an unordered protein) led to the conclusion that clustering of alcohol molecules is an important factor that enhances the effect of alcohols on proteins and peptides. Based on dynamic light scattering studies on β-lactoglobulin A and phosphoglycerate kinase, Gast *et al.* [274] proposed that HFIP exerted its effect as a consequence of extensive preferential binding.

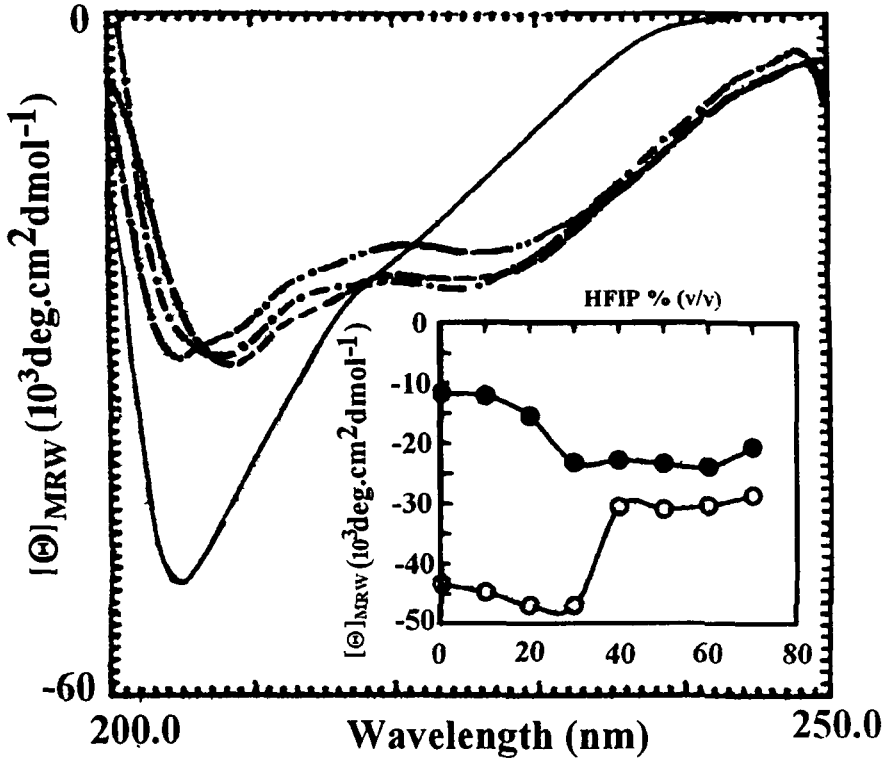


Fig.26. Far- UV circular dichroism spectra of *C. cajan* PI in the absence and presence of increasing concentrations (v/v) of hexafluoroispropanol (HFIP) 50% (——), 60% (—•—), 70 % (—••—).

Inset: Changes in $[\Theta]_{MRW}$ at 208 nm (O) and 222 nm (●) with increasing concentrations of hexafluoroisopropanol (HFIP).

Results & Discussion-99

Phaseolus aureus Roxb.

Syn. *Vigna radiata* (L.) Wilczek

Leguminosae

Mung bean, Black dhal, Black gram, Black mung, Golden gram, Gram bean, Green gram, Red mung bean, Urd

Kingdom:	Plantae
Division:	Magnoliophyta
Class:	Magnoliopsida
Order:	Fabales
Family:	Fabaceae
Genus:	Vigna
Species:	radiata

3. RESULTS AND DISCUSSION

3.2 *Phaseolus aureus* (Mung bean)

3.2.1 Purification of *P. aureus* inhibitor

The step-wise purification regime followed and the results obtained in terms of total protein, specific inhibitory activity, percent yield and fold purification are summarized in Table- XI. The elution profile of the *P. aureus* inhibitor from DEAE cellulose column is represented in figure 27A. Fractions containing inhibitory activity were pooled and further purified on a Trypsin-sepharose column (Fig.27B). The eluate was pooled, lyophilized and used thereafter for further studies. Protein content as well as inhibitory activity against trypsin, using N_{α}-Benzoyl L-arginine p-nitroanilide (BAPNA) as substrate was checked at each step. One unit of inhibitory activity was expressed arbitrarily in terms of decrease in absorbance by 0.001 units under the assay conditions. As is evident from the table, yield in case of trypsin inhibitory units (TIU) was 1.07 with a fold purification of approximately 12.5.

3.2.2 Molecular weight determination

In order to determine the molecular weight of the *P. aureus* inhibitor, SDS-polyacrylamide gel electrophoresis was performed under reducing conditions. Figure 28 shows the plot of log M (molecular weight of marker proteins) versus Rm (relative mobility of marker proteins on gel). The gel electrophoresis profile of the crude homogenate (lane1), 50-70% ammonium sulfate fraction (lane 2), ion-exchange fraction (lane 3), affinity purified inhibitor (lane 4) and marker proteins (lane 5) is shown in the inset of figure 28. The molecular mass of the *P. aureus* inhibitor, as determined by this method corresponds to approximately 13,600.

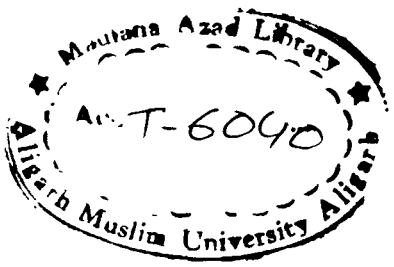


Table XI

Purification table of *P. aureus* proteinase inhibitor

Step	Total protein (mg)	Total Inhibitory Activity (IU[a])	Specific Inhibitory Activity (IU/mg)	Purification (fold)	Yield (%)
1. Homogenate	1054	210800	200	1	100
2. 50-70% Ammonium Sulphate Fraction	44	11918	272	1.36	5.7
3. DEAE – Celluose chromatography	6.8	2646	389	1.95	1.2
4. Affinity chromatography	0.9	2250	2500	12.5	1.07

[a] **Unit definition:** One trypsin unit = ΔA_{410} of 0.001 per min with BAPNA as substrate at pH 8.2 at 37°C. Reaction volume = 7 ml (1 cm light path). Inhibition thereof = IU

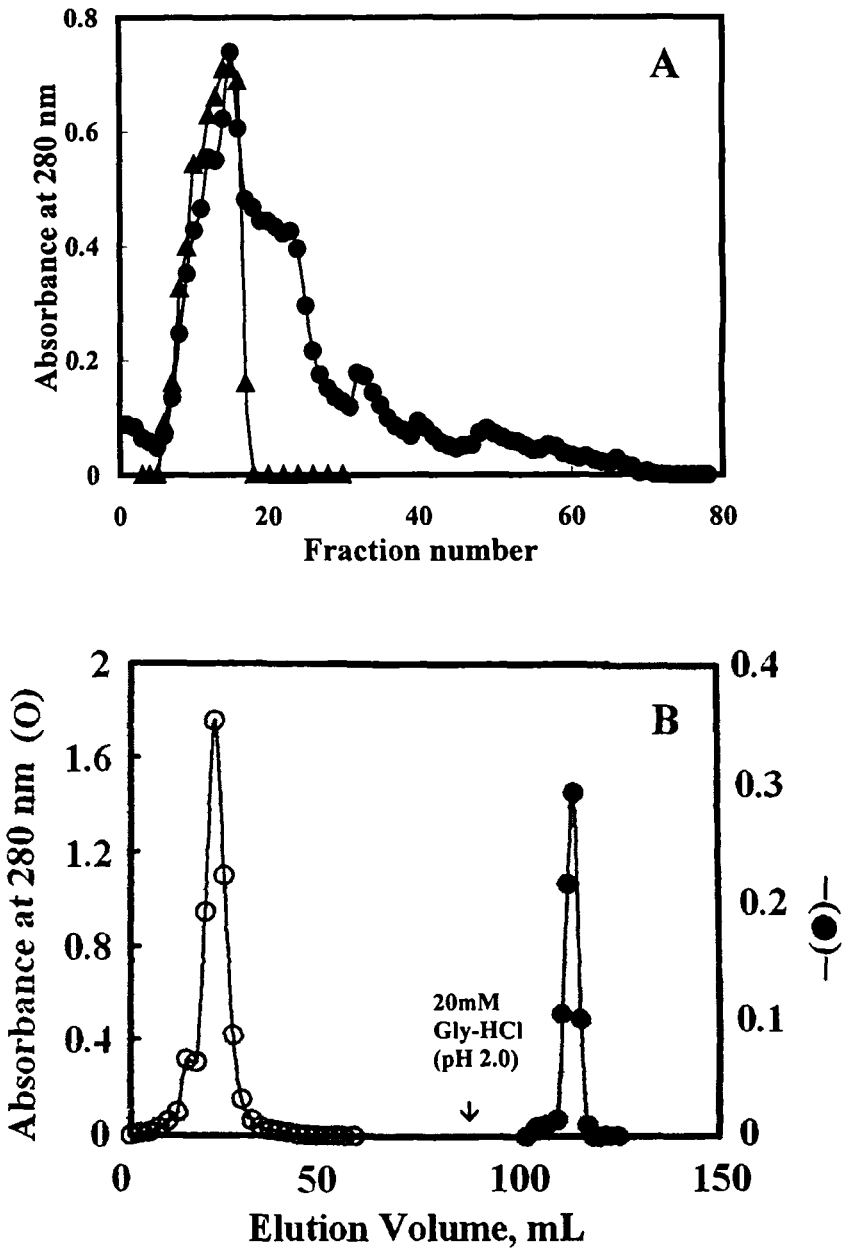


Fig.27. **(A)** Elution profile (•) of *P. aureus* inhibitor on a DEAE-Cellulose chromatography column (2.2 x 9.9 cm) equilibrated with 20 mM Tris-HCl buffer (pH 8.2). The PI was eluted in a linear gradient of 0 - 1M NaCl in the equilibration buffer. (▲) represents the inhibitory activity. [Fraction size=3mL/fraction].

(B) Washthrough (unbound) (o) protein and elution profile (•) of *P. aureus* PI on a Trypsin-Sepharose chromatography column (2.2 x 10 cm) equilibrated with 20mM Sodium phosphate buffer (pH 7.0). The PI was eluted with 20mM Glycine-HCl buffer, pH2.0.

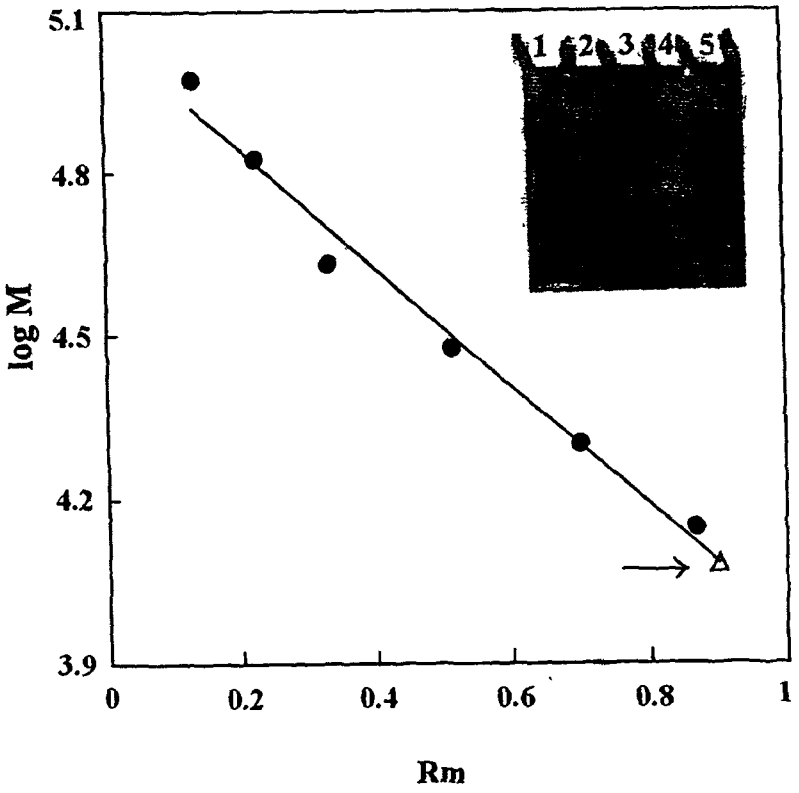


Fig.28. Molecular weight determination by SDS-PAGE: Log M versus relative mobility (Rm) of molecular weight markers: Phosphorylase b (Mr 94,000), Serum albumin (Mr 67,000), Ovalbumin (Mr 43,000), Carbonic anhydrase (Mr 30,000), Soybean trypsin inhibitor (Mr 20,000) and Bovine α- lactalbumin (Mr 14,000). Arrow indicates the position of *P. aureus* inhibitor (Δ).
Inset: SDS-Polyacrylamide gel electrophoretogram of crude homogenate (lane 1), 50-70% ammonium sulfate fraction (lane 2), ion-exchange chromatography fraction (lane 3), affinity eluate (lane 4) and molecular weight markers (lane 5).

3.2.3 Enzyme Inhibition Assay

The *P. aureus* inhibitor was found to specifically inhibit trypsin and α-chymotrypsin. Caseinolytic activity of papain was not inhibited by the inhibitor suggesting that the inhibitor is specific for serine proteinases and it does not inhibit cysteine proteinases. The fact that certain inhibitors are specific for a particular mechanistic class of proteases is attributed to the presence of specific amino-acid residue (termed the active-site residue, P_1) on the inhibitor that allows for complimentary binding of the inhibitor to the enzyme. The reactive site residue P_1 generally corresponds to the specificity of the cognate enzyme. Thus inhibitors with P_1 Lys and Arg tend to inhibit trypsin and trypsin-like enzymes. Figure 29A depicts the inhibition of amidolytic activity of trypsin by the *P. aureus* inhibitor. The curve shows a progressive decrease in the residual enzyme activity at increasing inhibitor to enzyme ([I]/[E]) molar ratio. The inhibition profile slopes off near [I]/[E] molar ratio corresponding to 1.0 suggesting a binding stoichiometry of 1:1 i.e. one molecule of inhibitor binds per molecule of enzyme. The proteolytic activity of trypsin was maximally reduced by ~65%.

In case of α-chymotrypsin (Fig.29A) almost 35% reduction in activity was observed. Greater reduction in the activity of trypsin as compared to α-chymotrypsin clearly indicates that the inhibitor is more effective against trypsin. The stoichiometry of binding to α-chymotrypsin is approximately 1:2. Several inhibitors have been reported that exhibit inhibitory activity towards both trypsin and a-chymotrypsin separately or simultaneously. In the former case, the binding site for both enzymes coincides; whereas in the latter case, separate binding sites are proposed to exist and accordingly such inhibitors are termed as 'bi-headed' [275].

Inhibition of α-amylase activity is depicted in figure 29B. The decrease in absorbance at 550 nm (in the presence of inhibitor) with respect to control (in the absence of inhibitor) was measured and expressed as percent of residual enzyme activity. As shown in the figure, the α-amylase activity is decreased by approximately 60% at inhibitor concentrations as low as 0.5 microgram. The dual specificity of the *P. aureus* inhibitor towards two different classes of enzymes (a proteinase and an amylase)

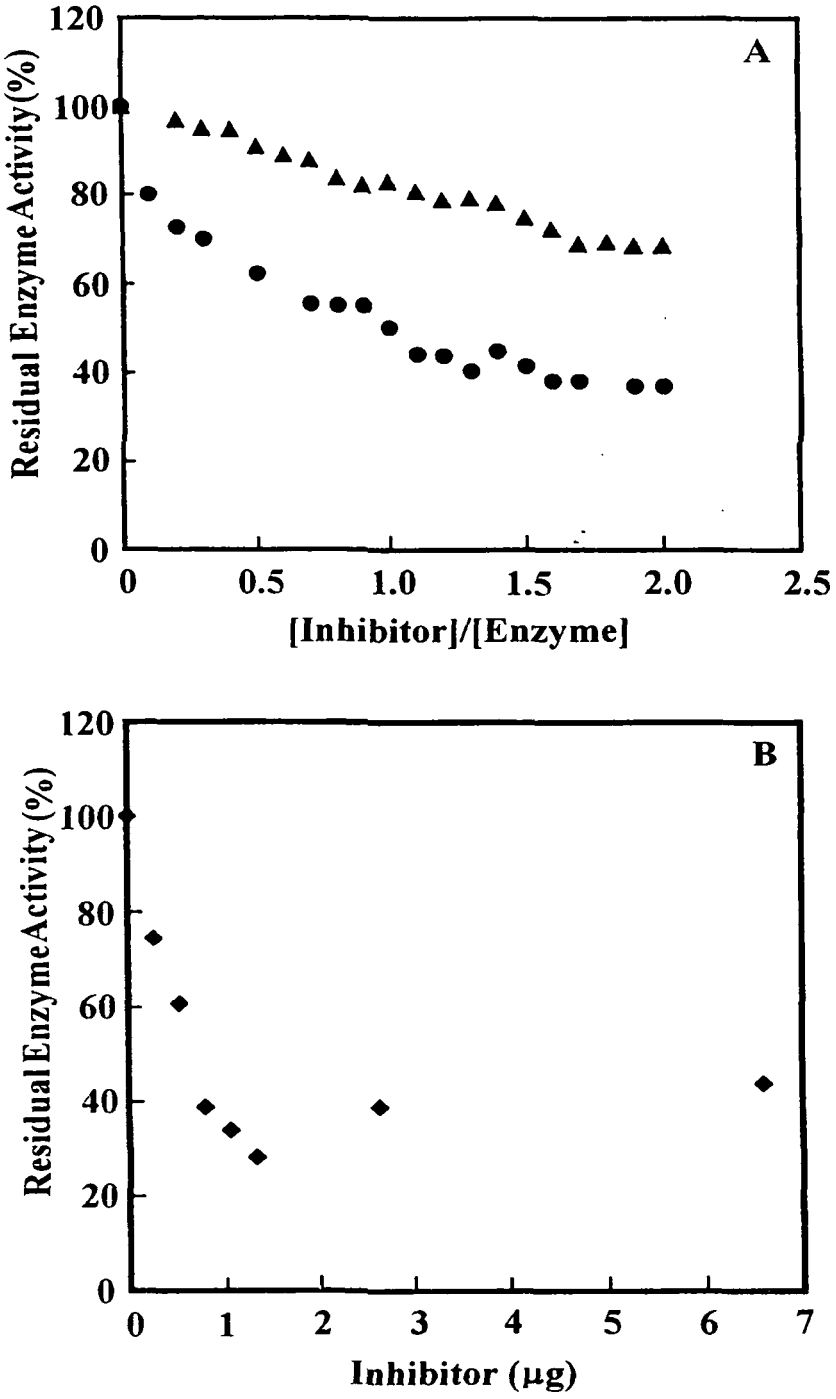


Fig.29. Enzyme inhibition. (A) Percent residual enzyme activity for trypsin (●) and α-chymotrypsin (▲) in the presence of varying concentrations of *P. aureus* inhibitor.

(B) Percent residual enzyme activity for α-amylase (◆) in the presence of varying concentrations of *P. aureus* inhibitor.

classifies it as a 'bi-functional' inhibitor [276] and will henceforth be termed as *P. aureus* proteinase/amylase inhibitor.

3.2.4 Estimation of tryptophan (Trp), tyrosine (Tyr) and cysteine (Cys)

Figure 30 represents the absorption spectrum of *P. aureus* proteinase/amylase inhibitor in the wavelength range 320-260 nm. As can be seen in the figure, the inhibitor shows a wavelength maximum at 280 nm indicative of the presence of aromatic amino acids. A protein generally, depending on its amino-acid composition, has a broad, featureless UV absorption spectrum with peaks in the 275nm to 290nm region. This peak arises from multiple contributions from tyrosine, tryptophan and phenylalanine residues. Tryptophan has a much greater absorption than the other amino-acids at these wavelengths. The number of tryptophans calculated according to the method of Spande and Witkop was approximately 1.36 (Table XII).

Ionization of tyrosyl residues in *P. aureus* proteinase/amylase inhibitor in the presence of increasing concentration of alkali (varying pH from 6.0-13.0) was performed under denaturing conditions in order to measure the total ionizable tyrosines. Figure 31 depicts the alkaline transition curves obtained under denaturing conditions. The ionization of tyrosine is accompanied by both an intensification of the spectrum and a shift of the maximum from 278 to 296nm. The alkaline difference spectrum (pH 13.0-6.0) of *P. aureus* inhibitor depicted in inset of figure 31, shows two peaks at 244 nm and 296 nm which is characteristic of the tyrosinate ion. pH induced difference spectra in the 250-300nm region have been ascribed to vicinal charge effects, change in polarity and polarizability of the chromophore environment, breaking and formation of side chain hydrogen bonds involving tyrosyl residues or simply ionization of tyrosyl residues at high pH. However, the main spectral changes observed with tyrosine containing proteins in the alkaline pH are due to ionization of tyrosyl residues [277]. In order to calculate the number of ionized tyrosyl residues, the maximum change in the molar extinction at 296 nm was measured and divided by 2300 cm^2/mole, the $\Delta\varepsilon$ of free tyrosine residue. Estimation of tyrosines by this method yielded 7.59 ionizable tyrosine residues in *P. aureus* inhibitor (Table XII).

The sulfhydryl groups in *P. aureus* proteinase/amylase inhibitor were titrated against DTNB under native as well as denaturing conditions. Under native conditions,

one free sulfhydryl group was detected. The results obtained under denaturing conditions are expressed in figure 32. As can be seen in the figure, the absorbance at 410 nm increases with increase in the ratio of [DTNB]/[Inhibitor] and the inflection point in the curve occurs at ~5.0 indicating the presence of a total of ~5 sulfhydryl groups. This suggests the presence of atleast two disulfide linkages in *P. aureus* inhibitor. The proposed molar extinction coefficient as calculated by the proposed equation by Pace et al. [230] was approximately 19,039.

The k_{ass} and K_i of the inhibitor for trypsin as calculated by the given equations (Methods) under pseudo first-order conditions were ~$3.1 \times 10^4 \pm 0.4$ $M^{-1}s^{-1}$ and ~ 3.5 nM respectively.

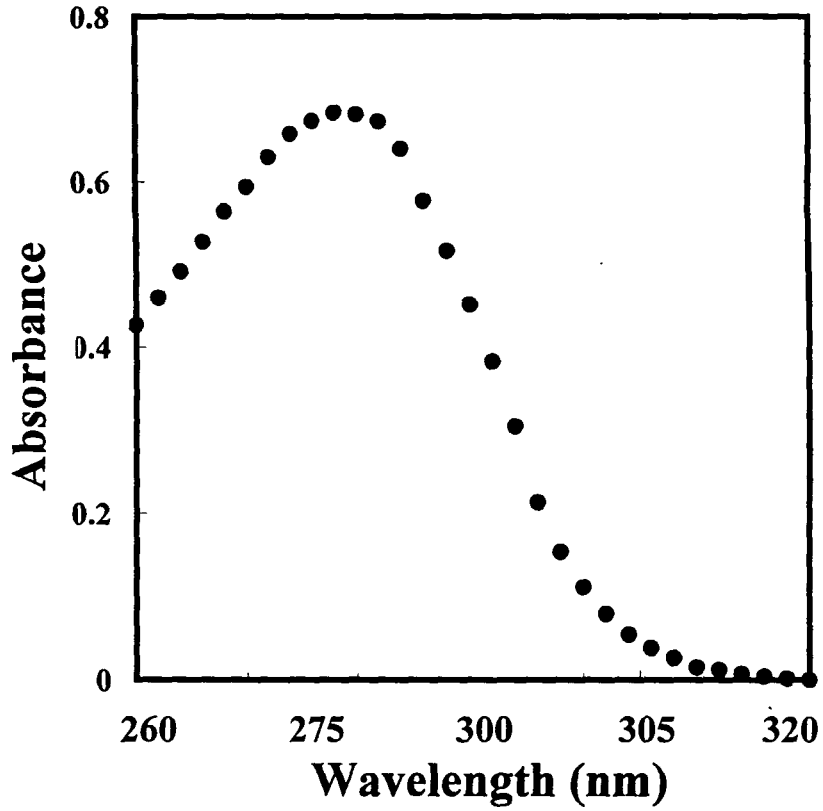


Fig.30. Absorption spectrum of *P. aureus* inhibitor depicted in the wavelength range 320-260 nm.

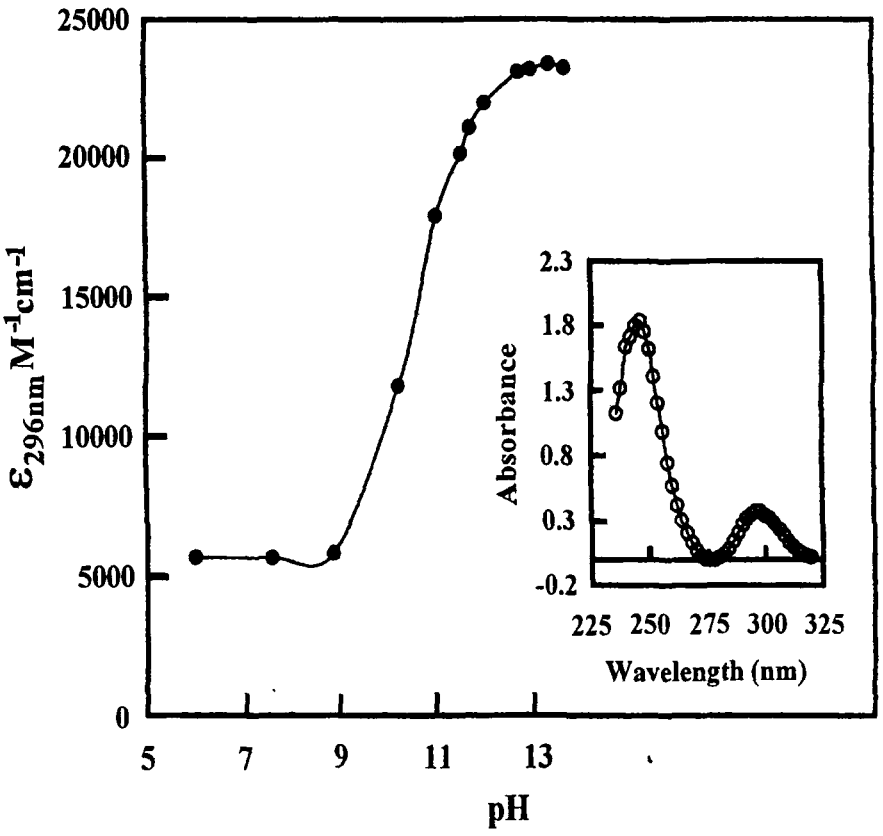


Fig.31. Tyrosine ionization. Alkaline transition curve of denatured *P. aureus* inhibitor. **Inset:** Alkaline difference spectrum of *P. aureus* inhibitor (pH 13.0 - 6.0).

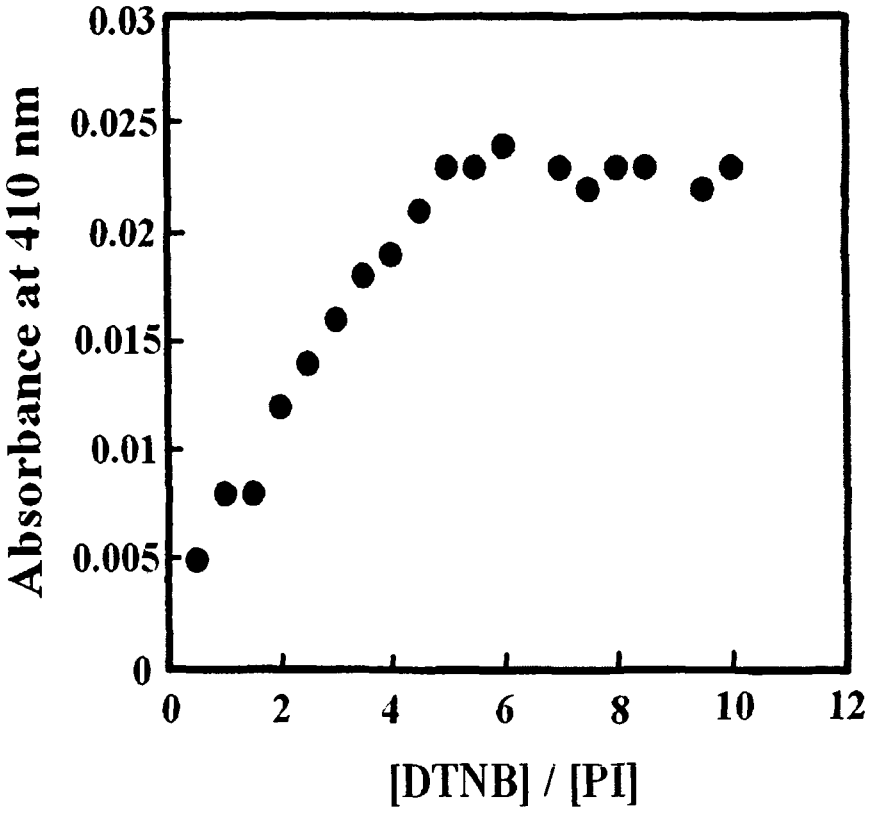


Fig.32. Sulfhydryl estimation. Plot of A_{410} versus [DTNB]/ *P. aureus* inhibitor. Fixed concentration of inhibitor was titrated against increasing molar excess of DTNB.

Table XII

Estimation of tryptophan, tyrosine and sulfhydryl content

Amino-acid	Estimated number	Method of Analysis
Tryptophan (Trp)	1.36 ± 0.1	Spande & Witkop [226]
Tyrosine (Tyr)	7.59 ± 0.2	Qasim & Salahuddin [227]
Cysteine (Cys)	5.20 ± 0.2	Habeeb [229]

3.2.5 Stability studies

The effect of varying pH treatments to the *P. aureus* proteinase/amylase inhibitor was examined by preincubating the inhibitor at the respective pH for 30 min prior to measuring its residual inhibitory activity. The inhibitor was observed to be quite stable in the pH range studied retaining about 70% of its activity at pH 1.0 and nearly 65% at pH 13.0 (Fig.33). The decrease in trypsin inhibitory activity observed at extremes of pH values suggests the prevalence of polar amino acids (eg. Lys/Arg at the reactive P_1 site) in the inhibitor that are affected by changes in pH and are required to maintain its trypsin inhibitory activity.

The residual trypsin inhibitory activity of *P.aureus* inhibitor after pretreatment of the inhibitor to different temperatures for 30min (Fig.34) shows no significant change in inhibitory activity. The rice cysteine proteinase inhibitors are a well-studied group that are reportedly heat-stable [31]. The soybean Kunitz trypsin inhibitor (SKTI) and trypsin/chymotrypsin inhibitor from chick pes (*Cicer arietinum*) are other stable proteins showing remarkable resistance to both thermal and acid denaturation [252,278,279].

The stability of the inhibitor towards tryptic cleavage for varying time periods was also assessed and results are shown in figure 35. As clearly seen in the figure, the PI retains ~80% of its proteolytic inhibitory after exposure to trypsin for 60 minutes. However, the amylase inhibitory activity is decreased after 10 min. and severely affected after 20 min. of treatment with trypsin. A possible explanation could be that the modified inhibitor (reactive site peptide bond cleaved; [40]) retains its inhibitory activity for proteinases as also reported previously [280]. However, the region of inhibitor affected by tryptic proteolysis presumably contained the site for α-amylase binding; hence the loss of inhibitory activity towards α-amylase.

3.2.6 Circular Dichroism conformational studies

To get an insight into the tertiary conformation and secondary structural elements of the *P. aureus* proteinase/amylase inhibitor, CD conformational studies were performed in the wavelength range 300-250 nm and 250-200 nm respectively. Curve 1 in figure 36A shows the near-ultraviolet circular dichroic spectrum of the inhibitor at neutral pH. A positive band near 255 nm, a weak shoulder around 268 nm and a strong trough with minima at 276 nm and 283 nm are the characteristic features observed. Since the

spectrum in the aromatic absorption region is strongly dependent on the number and environment of aromatic-acids *viz.* tryptophan, tyrosine and phenylalanine, the observed features are attributed to the presence of these amino-acids in the inhibitor. Figure 36B (curve 1) depicts the far- ultraviolet CD spectrum of *P.aureus* proteinase/amylase inhibitor. Negative ellipticities at 222 nm and 208 nm strongly suggest the predominance of ordered α-helical structure. Although most small-molecular weight Bowman-Birk inhibitors [242] as well as a few Kunitz inhibitors [8] reportedly possess aperiodic structure, several inhibitors are known to have a regular backbone secondary structure [281].

Changes in tertiary as well as secondary conformation at pH 2.0 and 10.0 were probed using circular dichroism spectroscopy (Figs. 36A and B). Near-UV CD spectrum shows marked changes in spectral features; the increase in negative dichroism being more pronounced at acidic pH. Far-UV CD also shows decrease in helical structure as evidenced by the decrease in MRE values at 208 and 222 nm. At acidic pH, far–UV CD shows less pronounced changes, however, secondary structural alterations are significant at pH 10.0 with a greater percentage of ordered helical structure getting converted to unordered structure (decrease in the negative CD band at 222 nm and the appearance of a negative extremum at ~200 nm with increase in temperature). Thus, conformational studies also indicate the importance of certain key residues (eg. Asp or Glu; Lys or Arg) that are important for maintaining protein structure and function.

Figure 37A represents changes in $[\Theta]_{MRW}$ values at 324 nm (reflective of tertiary structure) and 222 nm (a probe for helical structure) as a function of temperature. The *P. aureus* proteinase/amylase inhibitor exhibits a two-state transition with T_m at ~ 65°C. The far–UV CD spectral features at different temperatures are shown in figure 37B. As can be seen in the figure, the protein shows a gradual 'helix to unordered' transition as the temperature is raised from 30°C to 90°C. This suggests that the PI possesses some degree of flexibility since the temperature induced conformational changes are reversed and the protein retains its function under thermal stress. This stability towards thermal denaturation could be attributed to the likely presence of disulfides.

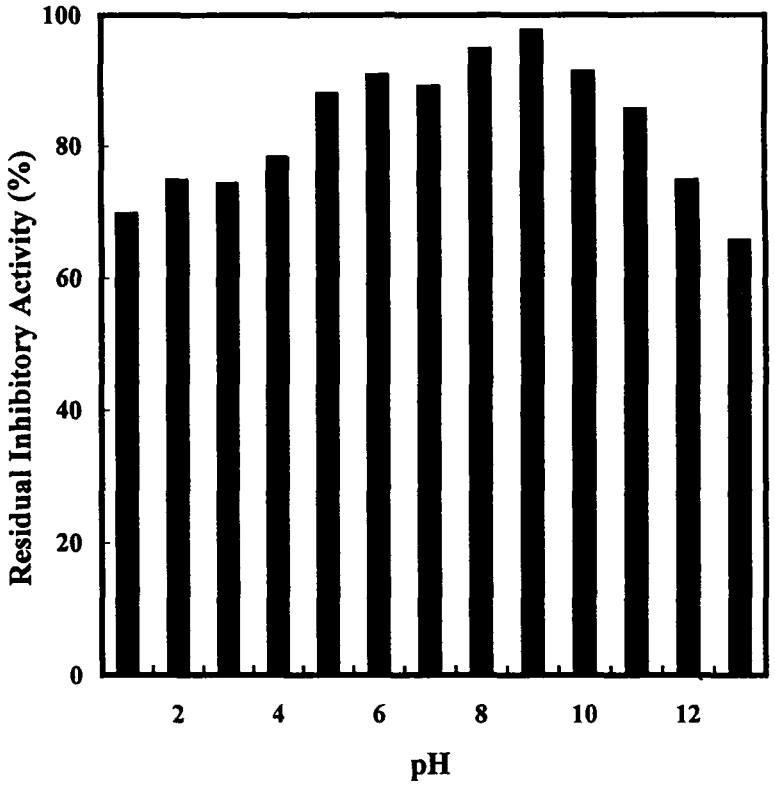


Fig. 33. Effect of pH on residual inhibitory activity of *P. aureus* inhibitor.

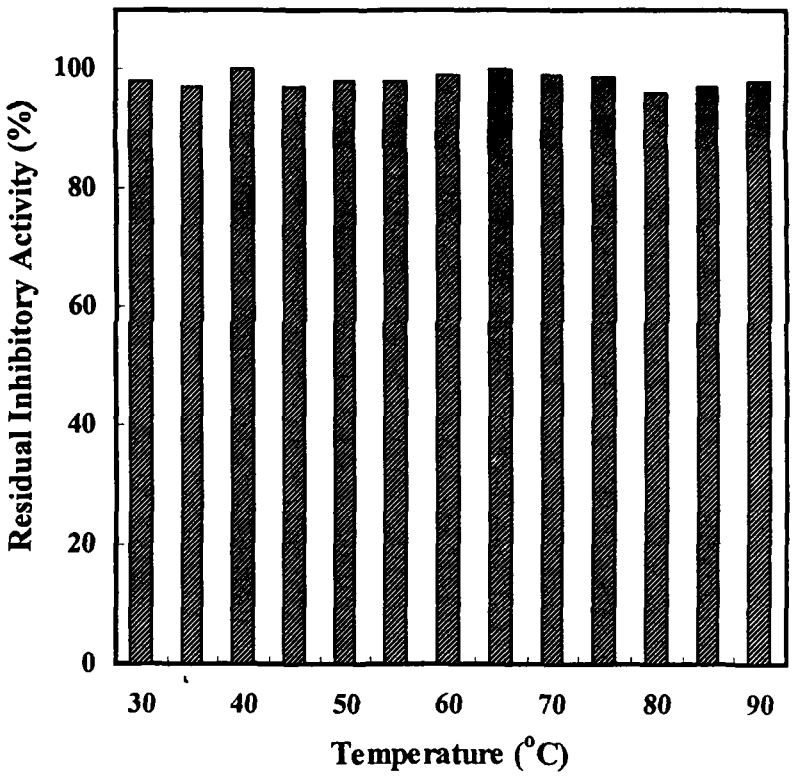


Fig. 34. Thermal denaturation. Percent residual inhibitory activity of *P. aureus* inhibitor versus temperature.

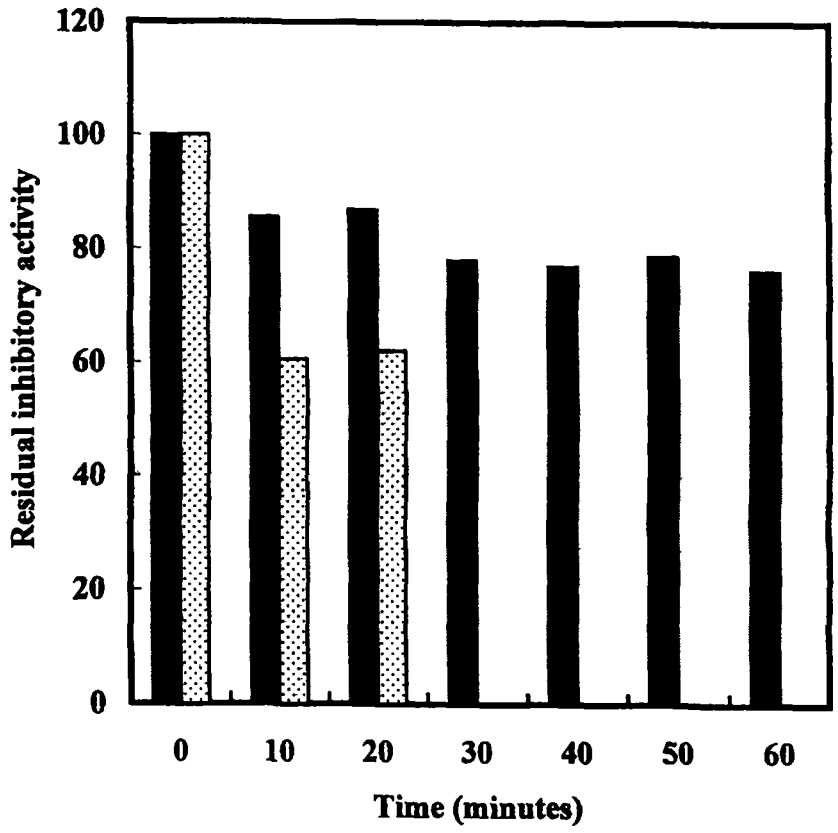


Fig.35. Effect of proteolysis on inhibitory activity. Residual inhibitory activity of *P. aureus* inhibitor after treatment to trypsin for varying time periods. Filled bar represents trypsin inhibitory activity and dotted bar represents amylase inhibitory activity.

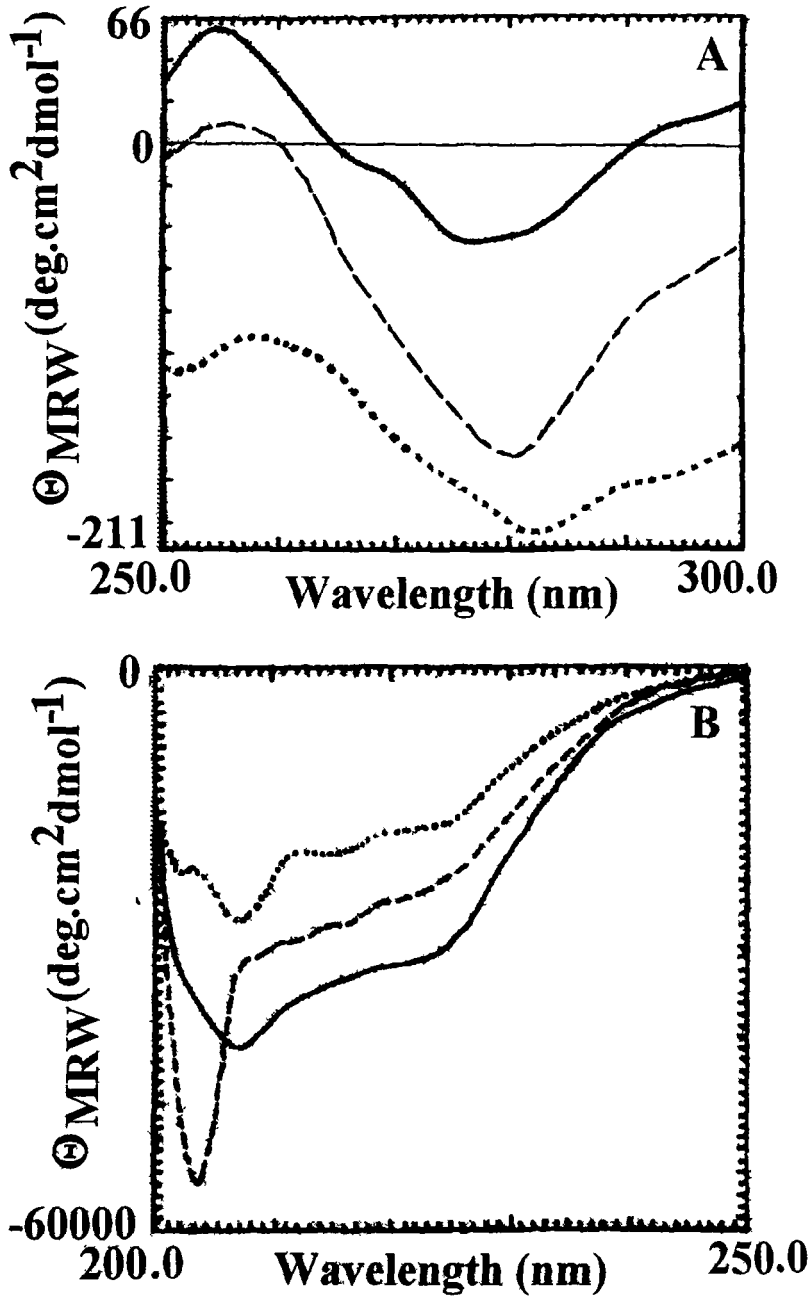


Fig. 36. Effect of pH on conformation. (A) Near-UV circular dichroism. Curve (——) represents *P. aureus* inhibitor at pH 7.0, curve (— —) at pH 10.0 and curve (••••) at pH 2.0.

(B) Far-UV circular dichroism. Curve (——) represents *P. aureus* inhibitor at pH 7.0, curve (— —) at pH 10.0 and curve (••••) at pH 2.0.

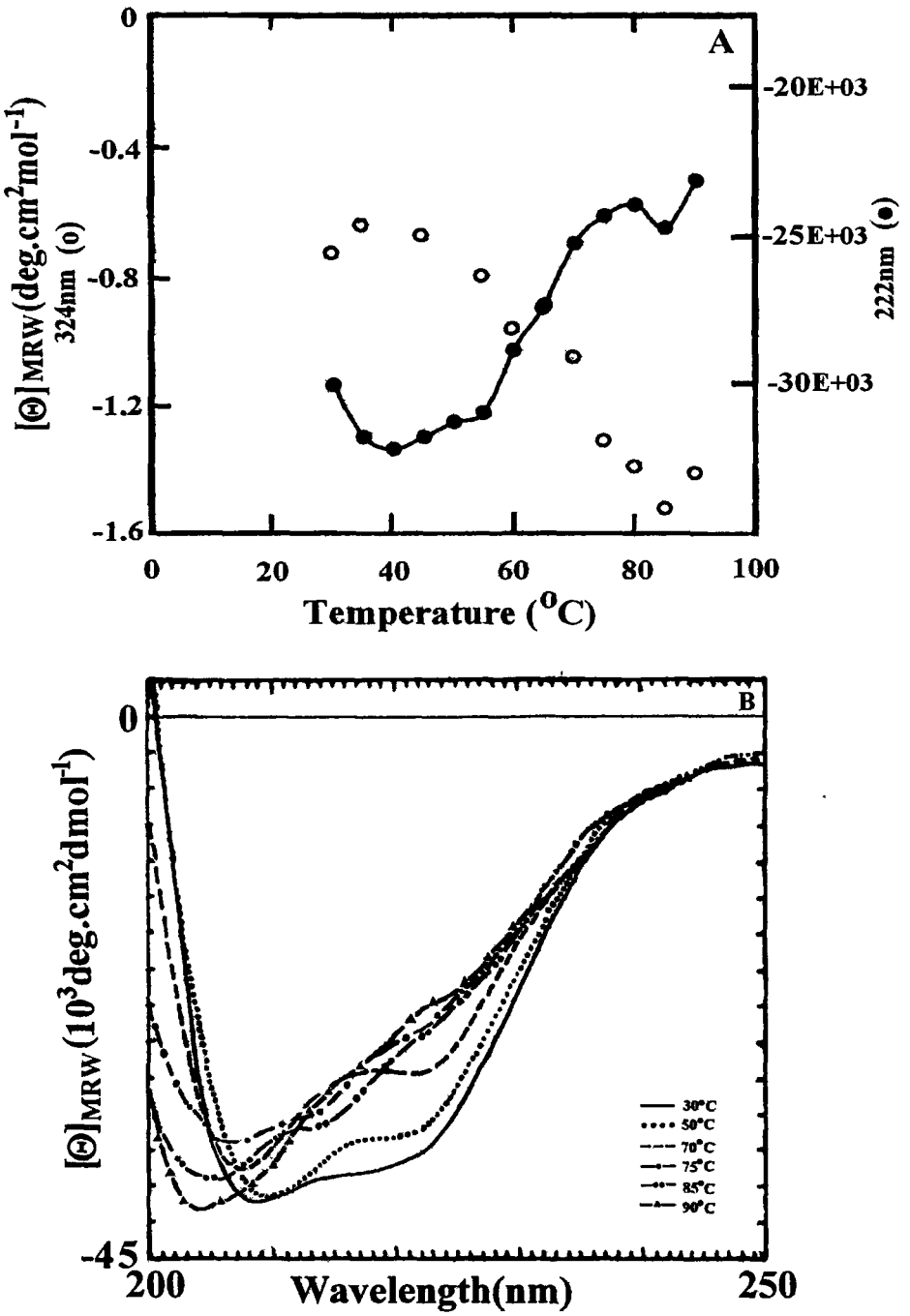


Fig. 37. Thermal denaturation. **(A)** Changes in $[\Theta]_{MRW}$ of *P. aureus* proteinase/amylase inhibitor at 324 nm and 222 nm as a function of temperature.
(B) Far-ultraviolet CD spectra of *P. aureus* inhibitor at various temperatures.

3.2.7 N-terminal sequence

The N-terminal sequence of the isolated bi-functional inhibitor is as follows:

Phe-Pro-Glu-Arg-Glu-Glu-Gln-Glu-Ser-Gln

Two sequences of Bowman-Birk type inhibitors reported previously are aligned with this sequence for comparison in Table-III. The reported sequences are actually of the same protein, a Bowman-Birk type trypsin inhibitor since the sequence of the protein with Accession number P01062 [282,283] is to be found in the sequence of the other protein with Accession number Q7Y1U3 [284], suggesting that the latter is the unmodified precursor sequence. As clearly evident from the table, the new sequence does not match either of the two at their N-termini nor is it to be found anywhere in the rest of the sequence. Hence, we can safely say that it is a novel protease/amylase inhibitor being reported from the mung bean, *Phaseolus aureus* Roxb. (*Vigna radiata* (L.)Wilczek)

Table XIII

N-terminal sequence of isolated *P. aureus* inhibitor aligned with Bowman-Birk type trypsin inhibitor[a] and Mung bean trpsin inhibitor[b]

Entry Name	Accession umber	Protein Name	Source	Sequence (First 10 AA)	Ref.
IBB_PHAAU	P01062	Bowman-Birk type trypsin inhibitor	*Phaseolus aureus*	**SHDEPSESSE**	[282,283]
Q7Y1U3	Q7Y1U3	Mung bean trypsin inhibitor	***Phaseolus aureus***	**MMVLKVCVLV**	[284]
---------	---------	*Phaseolus aureus* proteinase/amylase inhibitor	***Phaseolus aureus***	**FPEREEQESQ**	

The sequences were retreived from [a]Swiss-Prot and [b]SRS-EMBL-EBI database.

3.2.8 Effect of detergents

Detergents are used extensively for solubilizing proteins from lipid membranes and other insoluble biological material and for maintaining the solubility of certain proteins in solution. It is therefore essential to recognize the detergents that will accomplish the task of maintaining solubility while not significantly compromising the native structure and consequently the biological function of the protein [285]. Earlier scattering and hydrodynamic data were mainly used to probe the structural changes in protein-detergent complexes. However, more recently, spectroscopic approaches have gained more attention. The effect of detergents on proteins depends on the nature of both the detergent and the protein component; in addition the environmental conditions are of importance [286].

Figure 38A shows the far-UV CD structural changes associated with the addition of SDS (ionic detergent) to the helical proteinase/amylase inhibitor from *Phaseolus aureus*. The already α-helical protein shows increase in $[\Theta]_{MRW}$ at 208 nm indicative of increase in helicity (see figure inset). The propensity of SDS to induce structure is clearly envisaged here. An increase in the other major component of alpha-helix, ellipticity at 222nm, has been reported earlier for myoglobin in the presence of increasing SDS concentration [273]. Figure 38B shows the fluorescence emission spectrum of inhibitor in the absence and presence of SDS. As seen in the figure, SDS causes a slight decrease in fluorescence emission intensity of the aromatic residues in the *P. aureus* inhibitor without any alteration in λ_{max}. Alkyl sulfates at very low concentrations, have an important role in electrostatic interactions whereas at high concentrations their hydrophobic nature is more dominant [287,288]. On interacting with the *P. aureus* inhibitor, our results indicate that SDS mainly affects the backbone secondary structure without affecting much the tertiary conformation of the protein.

Figure 39A depicts the far-UV CD spectra of *P. aureus* inhibitor in the absence and presence of sodium deoxycholate. It can be seen in the figure, $[\Theta]_{MRW}$ at 208 nm is increased and slightly blue-shifted. Figure 39B depicts the effect of the zwittergent, CHAPS on $[\Theta]_{MRW}$ values at 222 nm for the *P. aureus* inhibitor. As evident from the figure, increase in the detergent concentration leads to an increase in the ellipticity at 222 nm, suggesting enhanced helical structure in the presence of a non-denaturing detergent.

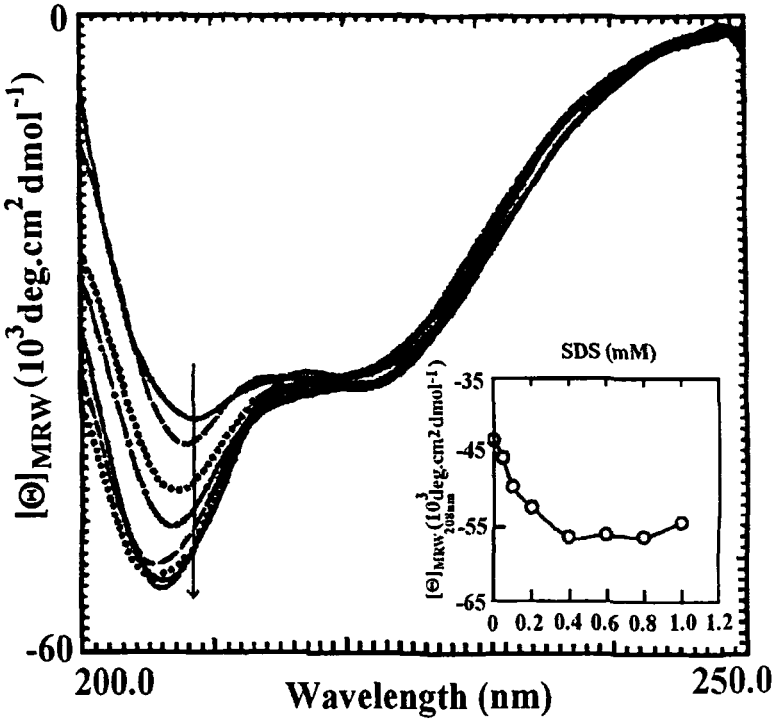


Fig.38. (A) Far-UV Circular Dichroism: CD spectra of *P. aureus* inhibitor in the absence and presence of increasing concentrations of sodium dodecyl sulphate. **Inset:** Changes in $[\Theta]_{MRW}$ at 208 nm (O) with increasing concentrations of sodium dodecyl sulphate.

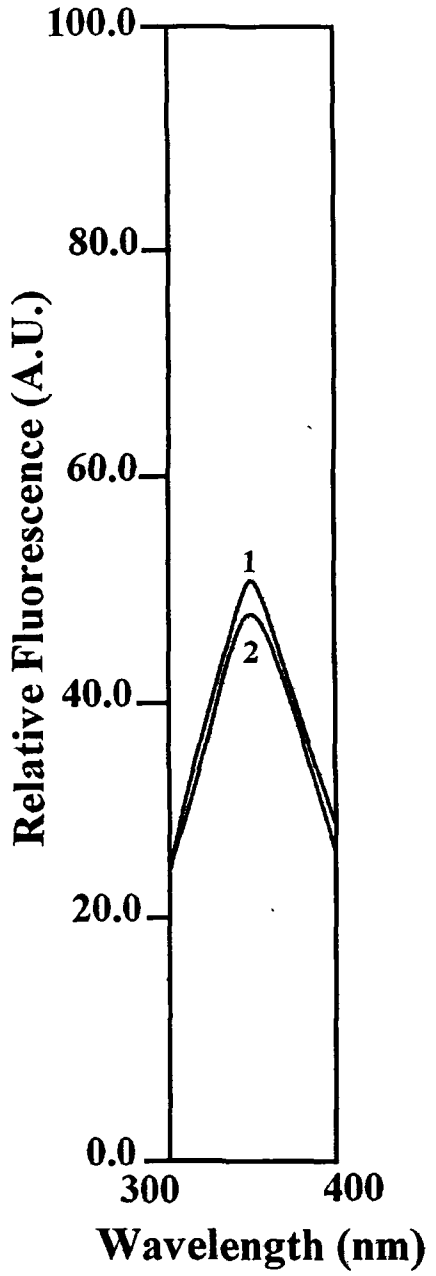


Fig.38. (B) Fluorescence emission spectrum of *P. aureus* inhibitor in the absence (curve1) and presence (curve2) of 1mM SDS.

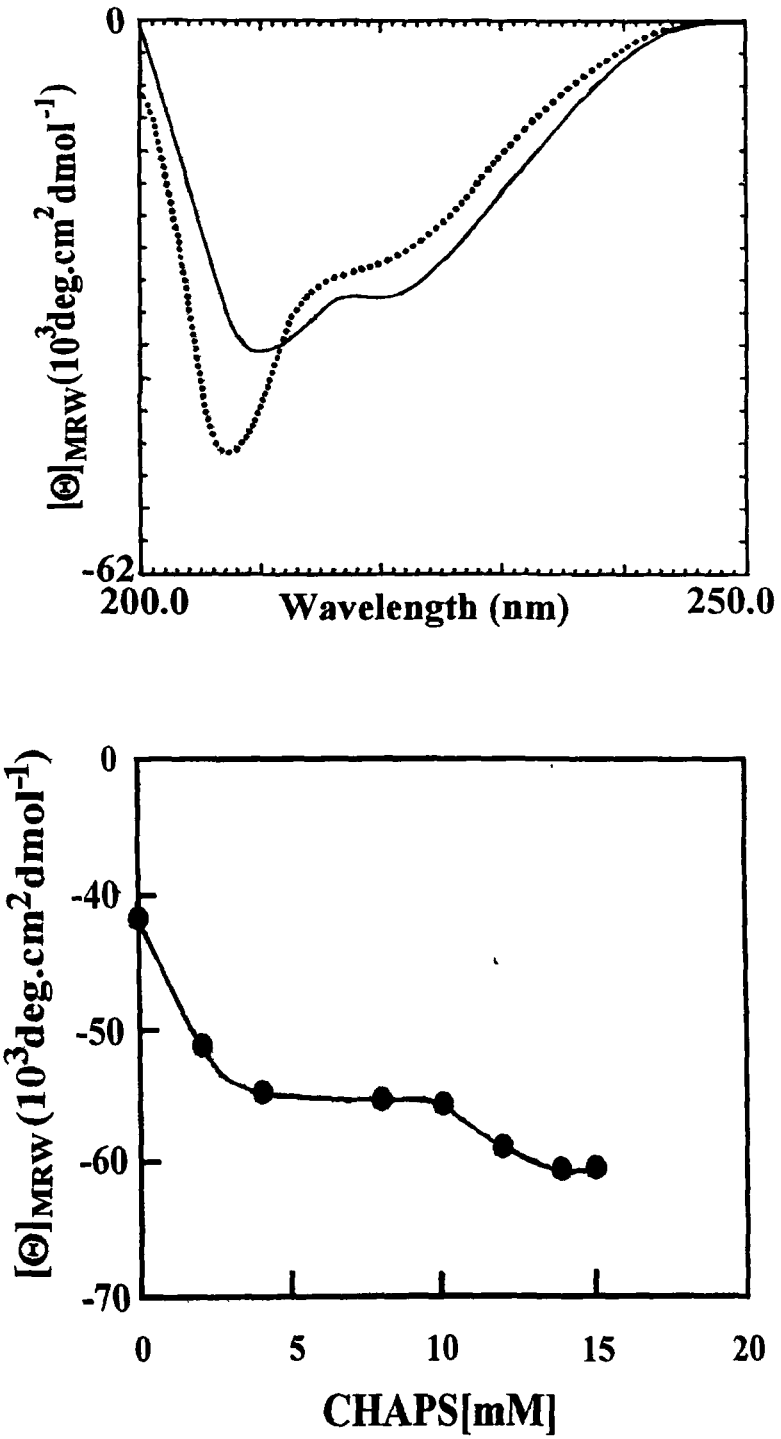


Fig.39. (A) Far-UV Circular Dichroism: Far –UV CD spectra for *P. aureus* inhbitor in the absence (——) and presence (••••) of 4 mM sodium deoxycholate. **(B) Far-UV Circular Dichroism:** $[\Theta]_{MRW}$ at 222 nm (●) for *P. aureus* inhibitor in the presence of increasing concentrations of CHAPS.

Based on previous studies on the interaction of hydrocarbons, anionic and neutral detergents and other amphiphilic substances [289-295], three distinct modes of interaction of detergents with proteins has been proposed [296]:

a) association with specific binding sites of native proteins

b) cooperative association between protein and a large number of detergent molecules without major conformational change

c) cooperative association with gross denaturation of the protein. The native structure is destroyed and replaced by an extended rod-like conformation with a moderately high content of α-helix, in which most of the hydrophobic residues are presumably exposed for association with the detergent. The cooperative transition (Fig.37) in the presence of SDS could be a consequence of this kind of interaction between detergent and protein.

3.2.9 Effect of Hexafluoroisopropanol

Figure 40 shows the effect of HFIP on the secondary structure of *Phaseolus aureus* inhibitor as seen by far-UV circular dichroism. A steady increase in α-helical content (increased ellipticity values at 208 as well as 222 nm) with increase in HFIP concentration upto 12% (v/v) is observed. Various mechanisms for the induction of helical structure in proteins by fluorinated alcohols have been proposed [270, 273,274]:

(i) HFIP causes the cooperative formation of micelle-like clusters that enhances the effect of alcohols on proteins and peptides.

(ii) HFIP exerts its effect as a consequence of extensive preferential binding (cluster formation being an accompanying phenomenon and not the primary cause)

Whatever be the exact mechanism, enhanced local hydrophobic interactions leading to an increased α-helix is an almost singular effect seen with such fluoroalcohols, as observed in case of the *P. aureus* inhibitor.

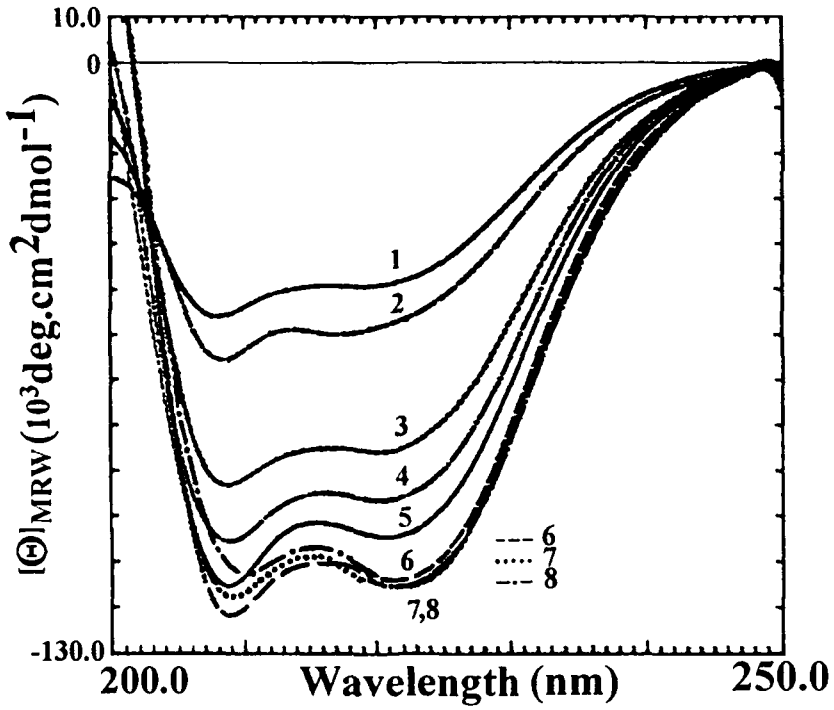


Fig.40. Far-UV Circular Dichroism: Far- UV circular dichroism spectra of *P. aureus* inhibitor in the absence and presence of increasing concentrations of hexafluoroispropanol (HFIP). 1,2,3,4,5,6,7 and 8 represent 0,2,4,6,8,12,16 and 20 % (v/v) HFIP respectively.

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LIST OF PUBLICATIONS / COMMUNICATIONS

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13. **Haq, S.K.,** Atif, S.M. and Khan, R.H. (2004) Proteinase inhibitors: A panacea? (Communicated)

14. **Haq, S.K.** and Khan, R.H. (2004) Effect of detergents and hexafluoroisopropanol on the conformation of a helical and a non-helical proteinase inhibitor. (Communicated)

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Characterization of a Proteinase Inhibitor from *Cajanus cajan* (L.)

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A protein proteinase inhibitor (PI) has been purified from pigeonpea *Cajanus cajan* (L.) PUSA 33 variety by acetic-acid precipitation, salt fractionation and chromatography on a DEAE-Cellulose column. The content of inhibitor was found to be 15 mg/20 g dry weight of pulse. The molecular weight of the inhibitor as determined by SDS-PAGE under reducing conditions was found to be about 14,000. It showed inhibitory activity toward proteolytic enzymes belonging to the serine protease group, namely trypsin and α-chymotrypsin. The inhibitory activity was stable over a wide range of pH and temperatures. Estimation of sulfhydryl groups yielded one free cysteine and at least two disulfide linkages. N-terminal sequence homology suggests that it belongs to the Kunitz inhibitor family. Structural analysis by circular dichroism shows that the inhibitor possesses a largely disordered structure.

KEY WORDS: *Cajanus cajan*; circular dichroism; fluorescence quenching; Kunitz inhibitor; N-terminal sequence; proteinase inhibitor.

1. INTRODUCTION

Proteinase inhibitors (PIs) comprise one of the most abundant classes of proteins in plants (Ussuf *et al.*, 2001). Most storage organs such as seeds and tubers contain 1 to 10% of their total proteins as PIs, which inhibit different types of enzymes (Ryan, 1981; Pearce *et al.*, 1982). These proteins have diverse biochemical functions, some of which include elimination of unwanted proteolysis (Laskowski and Kato, 1980), inhibition of intraerythrocytic development of Plasmodium falciparum (Rockett *et al.*, 1990), suppression of *in vitro* and *in vivo* replication of retroviruses (Vonderfecht *et al.*, 1988) and inhibition of growth of transformed cells (Schnebli and Burger, 1972; Chou *et al.*, 1974). The plant proteinase inhibitors of serine proteinases play a dominant role in natural plant defence and infection processes (Ramasarma *et al.*, 1994).

C. cajan (L.) Millsp. is one of the oldest food crops and ranks fifth in importance among edible legumes of the world (Salunkhe *et al.*, 1986). It is important in human nutrition as a rich source of dietary protein (Singh *et al.*, 1984). However, preharvest damage due to insect pests on developing seed and postharvest losses due to storage pests are severe (Reed and Lateef, 1990). Consequently, the amylase inhibitors (AIs) and proteinase inhibitors (PIs) have gained attention as possible tools of natural and engineered resistance against pests and pathogens (Ryan, 1990; Giri and Kachole, 1998). Besides this, increased exposure to dietary protease inhibitors has been shown to protect against some chemically induced animal tumors (Weed *et al.*, 1985; Mokhtar *et al.*, 1988) and it has been suggested that protection against cancer may be achieved through this avenue (Schelp and Pongpaew, 1988).

The legume proteinase inhibitors are classified into two main groups according to their size and cystine content. One group of inhibitors, the Bowman-Birk type, has molecular weights of around 8000 and relatively high cystine content. The other main category of inhibitors, the

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[3] Abbreviations: BAPNA, N_α-Benzoyl-L-Arg-*p*-nitroanilide; DTT, dithiothreitol; GPNA, Glutaryl-Gly-Gly-Phe β-naphthylamide; NATA, N-acetyl L-tryptophanamide; PI, proteinase inhibitor; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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Kunitz type, has molecular weights of around 20,000 and lower cystine content. A common feature of plant PIs is high level of S-S bridges that confer great stability to heat, pH extremes, and to hydrolysis by proteases. The uniqueness of Bowman-Birk type inhibitors is that they have very little ordered structure in them (α-helix and β-structure) but have high content of aperiodic structure, which offers greater flexibility to the molecule. However, the disulfide linkages in the protein minimize the conformational entropy and enhance the stability of these proteins (Ramasarma *et al.*, 1994). Crystal structure analysis of soybean trypsin inhibitor (Kunitz) has also revealed the absence of α-helix and most of the polypeptide chain is involved in approximate β-pleated sheet structures with little, if any, regular sheet structure. It is stabilized chiefly by hydrophobic interactions of short stretches of hydrogen bonded sheets and proved to be extremely stable against the denaturing effect of 6 M guanidine hydrochloride (Sweet *et al.*, 1974; Jirgensons *et al.*, 1969).

In continuation of the studies of proteinase inhibitors from Leguminosae seeds, the present paper describes the purification and some of the properties of a proteinase inhibitor from *C. cajan* pulse.

2. MATERIALS AND METHODS

2.1. Materials

C. cajan pulse (decorticated split cotyledons) was procured from the local market. Trypsin (3× crystallized), α-chymotrypsin (3× crystallized), Dithiothreitol and Hammersten casein were purchased from Sisco Research Laboratories, India. N-Acetyl-L-tryptophanamide (NATA), N_α-Benzoyl-L-Arg-*p*-nitroanilide (BAPNA) (Lot 37F0833), N-Glutaryl-Gly-Gly-Phe β-Naphthylamide (Lot 34F0402), DEAE cellulose (Lot 113H0367) and iodoacetamide were obtained from Sigma Chemical Co. (St. Louis, MO).

2.2. Methods

2.2.1. Purification of C. cajan *Inhibitor*

C. cajan pulse was soaked overnight in 0.02 M Tris-HCl buffer, pH 8.2 containing 2 mM $CaCl_2$. Thereafter it was homogenized in a blender for 2 to 3 min at moderate speed. The crude extract was filtered through a cheesecloth to remove the coarse residual matter. It was then centrifuged at 8000 rpm for 45 min at 4°C. The clear supernatant thus obtained was defatted with 0.1 M acetic acid (final pH of solution = 4.0). After overnight stirring, the extract was subsequently centrifuged at 8000 rpm for 45 min at 4°C. The supernatant was adjusted to pH 7.0 with liquor ammonia and raised to 50% saturation with ammonium sulphate. The precipitate was collected by centrifugation at 8000 rpm for 45 min, 4°C; dissolved in minimum amount of 2 mM $CaCl_2$ –20 mM Tris-HCl (pH 8.2) and dialyzed against the same buffer for 24 hr.

The dialyzed sample (10 ml) was applied to a DEAE cellulose column (2.2 × 9.9 cm) equilibrated with 2 mM $CaCl_2$ –20 mM Tris-HCl (pH 8.2) and the bound protein eluted with the same buffer containing 0.2 M NaCl. The fractions containing inhibitory activities against trypsin and α-chymotrypsin were pooled and used as the proteinase inhibitor.

2.2.2. Protein Estimation

Measurements of protein concentration were made by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

2.2.3. SDS-PAGE

SDS-PAGE was performed on 15% polyacrylamide slab gel under reducing conditions (Laemmli, 1970). Proteins were detected by staining the gel with 0.1% Coomassie brilliant blue R-250.

2.2.4. Trypsin and Chymotrypsin Inhibitory Activities

For the assay of trypsin inhibitory activity in *C. cajan* PI; increasing amount of PI was incubated with a fixed amount of trypsin in 1 ml of 2 mM $CaCl_2$ – 20 mM Tris-HCl (pH 8.2) at 37°C for 10 min. After addition of 5 ml of BAPNA in dimethyl sulfoxide, the residual enzyme activity was measured by monitoring the liberation of *p*-nitroaniline at 410 nm on an AIMIL Spectrochem MKII colorimeter.

α-Chymotrypsin inhibitory activity was assessed by incubating the enzyme with suitable quantities of the inhibitor in 2 mM $CaCl_2$ – 20 mM Tris-HCl (pH 8.2) for 10 min at 37°C, and thereafter assaying for residual enzyme activity using Hammersten casein or N-Glutaryl-Gly-Gly-Phe β-Naphthylamide as a substrate.

2.2.5. Determination of Tryptophan

Tryptophan content was assessed by the method of Goodwin and Morton (1946). The absorption spectrum

of *C. cajan* PI was measured in the wavelength range 300–220 nm in the presence of 0.1 M NaOH. The formula used was: $w = (A_{280} - x \cdot \varepsilon_y)/(\varepsilon_w - \varepsilon_y)$ where w was estimated tryptophan content in moles per liter; A_{280} was absorbance at 280 nm; ε_y and ε_w were molar extinction coefficients of tyrosine and tryptophan in 0.1 M alkali at 280 nm ($\varepsilon_y = 1576$ and $\varepsilon_w = 5225$).

Tryptophan content was also estimated by the method of Spande and Witkop (1972). The initial optical density of the *C. cajan* PI in 8 M urea at 280 nm was recorded and further decrease in optical density by the addition of a fixed aliquot (10 μl) of 10 mM stock solution of N-Bromosuccinimide. The addition was continued in this stepwise fashion until further increments lead to no further decrease in optical density, with due allowance for the small decrease expected from dilution. The minimum optical density was recorded and corrected for the volume increase due to the added reagent. The reagent itself and succinimide display negligible absorption at 280 nm.

2.2.6. *Estimation of Tyrosine*

Tyrosine content was estimated by the ionization of phenolic hydroxyl groups. The titration of the phenolic groups of the *C. cajan* PI was carried out spectrophotometrically (Qasim and Salahuddin, 1977; Salahuddin and Khan, 1997) at 30°C. Two identical solutions of PI at pH 6.0 and pH 13.0 were prepared and an alkaline difference spectrum of the PI was recorded in the wavelength region 236–320 nm. Extreme care was taken to ensure identical protein concentration in the two samples.

2.2.7. *Measurement of Total Sulfhydryl Content*

The total sulfhydryl content of the purified enzyme was measured by the method of Habeeb (1972), using DTNB in which the release of nitrothiobenzoate anion was measured by following the increase in absorbance at 410 nm. The protein preparation was denatured by 6 M Gdn. HCl and reduced by 0.025 M β-mercaptoethanol. After 2 hr the protein was precipitated with 5% trichloroacetic acid and precipitate resuspended in 8 M urea. Subsequently 2% SDS was added and the solution titrated against DTNB.

2.2.8. *Effect of Temperature and pH on C.* cajan *PI*

The thermal stability test of inhibitory activity was done in 20 mM $CaCl_2$ – 20 mM Tris-HCl (pH 8.2) by treating the PI sample for 30 min at various temperatures, *viz* 25°C, 30°C, 37°C, 56°C and 95°C in a thermostat. The samples were then adjusted to 37°C and checked for residual inhibitory activity.

The effect of pH on the inhibitory potential of *C. cajan* PI were also checked by preincubating the PI with the enzyme at the desired pH (2.0–10.0) for 10 min and then assaying for residual enzyme activity. Ten-millimole solutions of the following buffers were used to get the desired pH: Gly-HCl (pH 3.0–4.0), sodium acetate (5.0–6.0), sodium phosphate (pH 7.0–8.0) and Gly-NaOH (pH 9.0–10.0).

2.2.9. *Reduction of* C. cajan *PI*

The inhibitor (0.5 mg/ml) in 2 mM $CaCl_2$ –20 mM Tris-HCl (pH 8.2) was treated with DTT (2 mM final concentration) for 60 min at room temperature and the reaction stopped with 2-fold molar excess of iodoacetamide. After stirring for 30 min at 4°C, the reduced samples were dialyzed against the same buffer and used for activity measurements as well as quenching experiments.

2.2.10. *Fluorescence Titration Measurements*

Fluorescence quenching measurements were performed on a Shimadzu RF 540 spectrofluorometer equipped with a data recorder DR-3 using an excitation wavelength of 280 nm and emission wavelength range of 300–400 nm. *C. cajan* PI samples, native as well as reduced, and N-acetyl-L-tryptophanamide (NATA) were titrated with 2 M acrylamide and 1 M potassium iodide (containing 0.1 mM sodium thiosulphate to prevent oxidation and formation of I_3^-). Relative fluorescence intensity was recorded with progressive addition of 10 μl aliquots of the quenchers. Because acrylamide had intrinsic absorption at 280 nm the inner filter effect was corrected using the equation:

$$F_{corr} = F_{obs} \cdot 10^{A/2} \quad (1)$$

where A is the absorbance of the sample at 280 nm by the addition of acrylamide (Lakowicz, 1983).

The contribution of collisional quenching to the deactivation rate of an excited state fluorophore is given by the Stern-Volmer equation:

$$F_O/F = 1 + K_{SV}[Q] \quad (2)$$

where F_O and F are the fluorescence intensities in the absence and presence of a quencher at a molar concentration

of [Q], and K_{SV} is the Stern-Volmer constant which is the product of a collisional quenching rate constant and the excited state lifetime of the fluorophore in the absence of quencher. In a heterogenous population of fluorophores, such as tryptophan residues in a protein, the linear relationship between F_O/F and [Q] is generally not obeyed. In such species it is assumed that energy transfer between accessible and inaccessible fluorophores is negligible and that all fluorophores have identical absorptivities. The fluorescence quenching is described by the modified Stern-Volmer equation:

$$\frac{F_O}{F_O - F} = \frac{1}{f_a K_{SV}} \cdot \frac{1}{[Q]} + \frac{1}{f_a} \quad (3)$$

where f_a is the maximum fraction of the protein fluorescence accessible to quencher.

2.2.11. CD Spectroscopy

Circular dichroism measurements were made on a Jasco J-720 spectropolarimeter thermostated with a Neslab water bath precalibrated with *d*-10-camphorsulfonic acid. The measurements were made at 25°C using quartz cells of 1 mm path-length in the far-UV region and 1 cm path-length in the near-UV region. The path-lengths of the cell and protein concentration (0.2–0.5 mg/ml) were chosen to optimize the measuring conditions. Each spectrum was an average of two scans. Results are expressed in terms of $[\theta]_{MRW}$ (Schmid, 1990) where

$$[\theta]_{MRW} = \frac{\theta \times 100 \times MRW}{c \times d} \quad (4)$$

where θ is the measured ellipticity in degrees, c is the protein concentration in mg/ml, d is the path-length in cm, and MRW is the mean residue weight. The mean residue weight used was 110 in all cases.

2.2.12. Kinetics of Binding to Serine Proteinases

The kinetics of association of *Cajanus cajan* PI to trypsin and α-chymotrypsin were analyzed by measurements of the loss of enzyme activity in the presence of specific chromogenic substrates under pseudo first-order conditions. The enzymes were mixed with a large excess of inhibitor and samples withdrawn at timed intervals to measure the residual enzyme concentration [E]. The initial concentration of PI was varied from 0.1–1.7 μM for analyses with trypsin and from 0.08–8.6 μM for analyses with α-chymotrypsin and substrate hydrolysis did not exceed 10%. k_{ass} was calculated from the relationship:

$$k_{ass} = k_{obs}/[I]_o \quad (5)$$

where k_{obs}, (pseudo first-order rate constant) was obtained from the slope of a plot of ln[E] (ln[residual enzyme activity]) against t (time of sampling) and $[I]_o$ represents the inhibitor concentration.

Inhibition constants, K_i, for the binding of *C. cajan* PI to trypsin and α-chymotrypsin were determined from the equilibrium rates of substrate hydrolysis by the enzymes at different inhibitor concentrations. Apparent inhibition constants, $K_{i(app)}$ were obtained by least-squares regression of plots of the ratio between the inhibited and uninhibited rates of substrate hydrolysis against inhibitor concentration as given by

$$v_o/v_i = 1 + [I]_o/K_{i(app)} \quad (6)$$

where v_o represents the uninhibited rate and v_i, the inhibited rate of substrate hydrolysis. K_i, the true equilibrium constants were calculated after correction for substrate competition.

2.2.12. Protein Sequencing

The N-terminal sequence was determined on a Shimadzu PPSQ-10 automated protein sequencer using Edman degradation. Phenylthiohydantoin amino acids (PTH-AA) were detected at 269 nm after separation on a reverse-phase CR7A Chromatopac HPLC column (4.6 mm × 25 cm) from Shimadzu, under isocratic conditions, using 40% acetonitrile, 20 mM acetic acid and 0.014% sodium dodecyl sulfate as the mobile phase at a flow rate of 1.0 ml/min at 40°C.

3. RESULTS AND DISCUSSION

3.1. Purification of *C. cajan* Proteinase Inhibitor

The purification of *C. cajan* PI yielded 15 mg of PI from a 20-g dry weight of pulse. The details are summarized in Table 1. The elution profile of the PI from DEAE cellulose column is represented in Figure 1. SDS-polyacrylamide gel electrophoresis of the eluted *C. cajan* PI yielded a single band corresponding to a molecular weight of 14,000 (Fig. 2).

No appreciable amounts of tannins have been reported in pigeonpea but they do contain considerable amounts of polyphenolic compounds that inhibit digestive

Table 1. Purification of *C. cajan* Proteinase Inhibitor (PI)

Step	Total Protein (mg)	Yield (%)	Total Inhibitory Activity (IU[a])	Specific Inhibitory Activity (IU/mg)	Purification (fold)
1. Homogenate	1300	100	678600	522	1
2. Acid-precipitation	800	61.5	nd*	—	—
3. 50% Ammonium sulphate fraction	242	18.6	199625.8	824.9	1.58
4. DEAE – Cellulose chromatography	15	1.2	14800	986.7	1.89

[a] **Unit definition:** One trypsin unit = ΔA_{410} of 0.001 per min with BAPNA as substrate at pH 8.2 at 37°C. Reaction volume = 7 ml (1 cm light path). Inhibition thereof = IU.
* nd = not determined.

enzymes. Polyphenols constitute about 3–18.3 mg/g of pigeonpea seeds (Rao and Deosthale, 1982; Singh, 1988). To rule out the contribution of polyphenolic compounds to the inhibitory activity, some samples of *C. cajan* PI were passed through a Sephadex G-50 column (Gegenheimer, 1990). No alteration in the inhibitory activity was observed (results not shown).

3.2. Trypsin and Chymotrypsin Inhibitory Activities

The *C. cajan* PI was found to specifically inhibit trypsin and α-chymotrypsin. Caseinolytic activities of papain and stem bromelain were not inhibited by the inhibitor. Figure 3 depicts the inhibition of amidolytic activity of trypsin and caseinolytic activity of α-chymotrypsin by the *C. cajan* PI. The proteolytic activity of trypsin was reduced sharply at very low concentrations of *C. cajan* PI. Even at 2.5 μg/25 μg enzyme inhibition of enzyme activity was more than 80%. The maximal inhibition of trypsin was 86.9 ± 1.57. In case of α-chymotrypsin almost 50% reduction in activity was observed at 5 μg/25 μg enzyme. This was followed by a gradual decrease (up to 65% ± 0.35) in the residual enzyme activity. Greater reduction in the activity of trypsin as compared to α-chymotrypsin clearly indicates that the PI is more effective against trypsin.

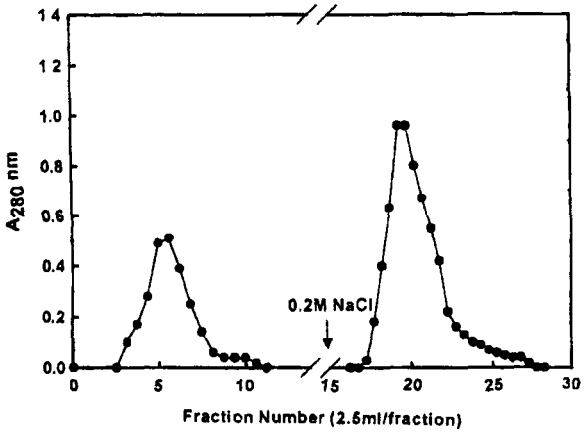

Fig. 1. Elution profile of *C. cajan* PI on a DEAE-Cellulose chromatography column (2.2 × 9.9 cm) equilibrated with 2 mM $CaCl_2$ – 2 mM Tris-HCl buffer (pH 8.2). The PI was eluted batchwise with 0.2 M NaCl in the equilibration buffer.

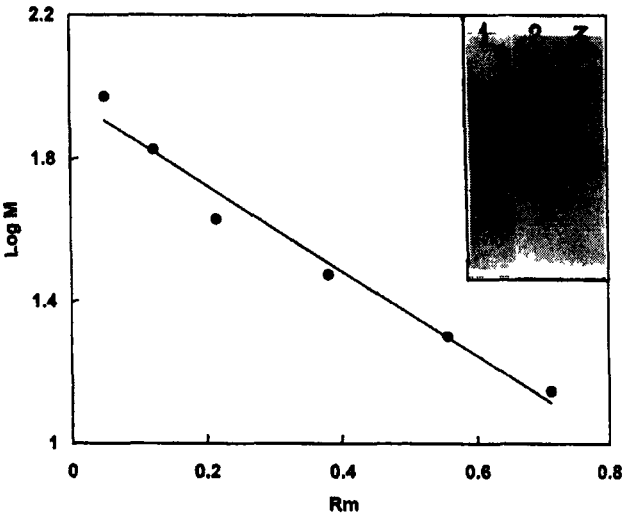

Fig. 2. Molecular weight determination by SDS-PAGE: Log M versus relative mobility (Rm) of molecular weight markers: Phosphorylase b (Mr 94,000), Serum albumin (Mr 67,000), Ovalbumin (Mr 30,000), Carbonic anhydrase (Mr 43,000), Soybean trypsin inhibitor (Mr 20,000) and Bovine α-lactalbumin (Mr 14,000). Inset: Electrophoretogram of the proteinase inhibitor from *C. cajan* (lane 3). Lanes 1 and 2 are crude homogenate and 50% ammonium sulphate fraction respectively.

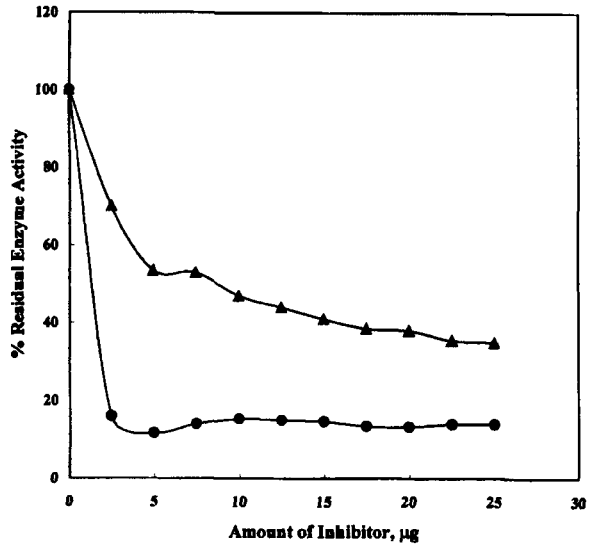

Fig. 3. Percent residual enzyme activity for trypsin (—●—) and α-chymotrypsin. (—▲—) in the presence of varying concentrations of *C. cajan* PI.

3.3. Estimation of Tyrosine, Tryptophan and Sulfhydryl Content

The number of tryptophans calculated according to the method of Goodwin and Morton were ~2 (Table 2). That determined by the method of Spande and Witkop was also ~2. A representative fluorescence emission spectrum of *C. cajan* PI is given in Figure 4A. In proteins containing all three amino acids, *viz.*, tryptophan, tyrosine and phenylalanine; the observed emission is due mainly to tryptophan. Proteins that contain no tryptophan, such as ribonuclease show the emission band due to tyrosine. The fluorescence emission spectra of *C. cajan* PI as shown in the figure clearly indicates an emission wavelength maximum (λmax) at 340 nm that is a characteristic feature of this amino acid (Teale and Weber, 1957). No tryptophan containing protein so far has been shown not to give tryptophan fluorescence. Therefore presence of indole fluorescence can be considered as good evidence that the protein is containing this amino acid.

Table 2. Estimation of Tryptophan, Tyrosine and Sulfhydryl Content

Amino acid	Estimated Number[a]	Method of analysis
Tryptophan (Trp)	2.0 ± 0.49	Goodwin & Morton
	2.1 ± 0.12	Spande & Witkop
Tyrosine (Tyr)	1.8 ± 0.03	Qasim & Salahuddin
Cysteine (Cys)	5.7 ± 0.12	Habeeb

[a] The results are obtained as a mean of three independent observations.

The alkaline difference spectrum (Fig. 4B) of *C. cajan* PI shows two peaks at 244 nm and 296 nm and an isosbestic point near 272 nm, which is characteristic of the ionization of phenolic hydroxyl groups of tyrosine. In order to calculate the number of ionized tyrosyl residues, the maximum change in the molar extinction at 296 nm was measured and divided by 2300 cm^2/mole, the Δε of free tyrosine residue. Estimation of tyrosines by this method yielded 1.8 ionizable tyrosine residues in *C. cajan* PI (Table 2).

The sulfhydryl groups in *C. cajan* PI were titrated against DTNB under native as well as denaturing conditions. Under native conditions, only one mole of DTNB reacted per mole of the protein suggesting one free sulfhydryl group. However, under denaturing conditions the absorbance at 410 nm was found to increase with increase in the ratio of [DTNB]/[*C. cajan* PI] and the inflection point in the curve (figure not shown for clarity) occurs at 5.0 indicating the presence of a total of ~5 sulfhydryl groups. This suggests the presence of at least two disulfide linkages in *C. cajan* PI.

3.4. Effect of Temperature and pH on *C. cajan* PI

The residual inhibitory activity of *C. cajan* PI was measured after pretreatment of the inhibitor at different temperatures for 30 min. The PI was quite resistant to temperature denaturation. Even after heating the PI at 95°C for 30 min, the residual inhibitory activity was approximately 85%. This is in agreement with an earlier report by Mulimani and Paramjyothi (1994) that the application of dry heat to the seeds and meal of red gram was not effective in inactivating the trypsin inhibitory activity (TIA) and chymotrypsin inhibitory activity (CIA). However, the inhibitory activity was lost upon boiling the crude extract at 100°C prior to ammonium sulphate fractionation.

The effect of pH on the activity profile of trypsin in the absence and presence of *C. cajan* PI was also tested. The PI was found to significantly inhibit the amidolytic activity of trypsin in the pH range 6.0–10.0 suggesting that the PI was also quite resistant to pH effects.

3.5. Reduction of *C. cajan* PI

The effect of 2 mM DTT treatment on the inhibitory activity of *C. cajan* PI was also checked. Table 3 shows that the inhibitory activity of PI decreases up to 18% upon reduction with DTT for 1 hr. This is indicative of the presence of disulfide linkages important for the structure and inhibitory activity of PI.

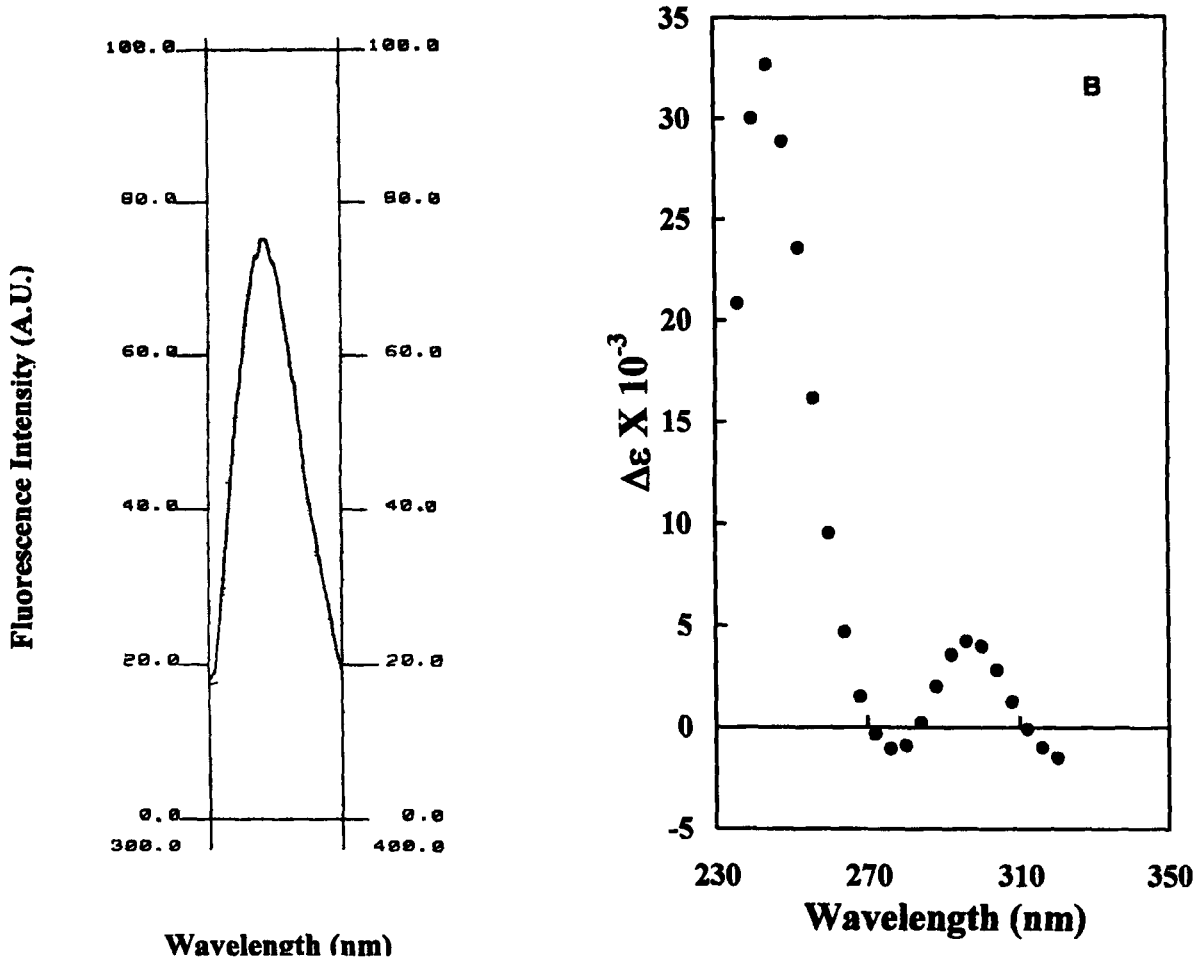

Fig. 4. (A) Fluorescence emission spectrum of *C. cajan* PI. $\lambda_{ex} = 292$ nm. (B) Alkaline difference spectrum of *C. cajan* PI in the wavelength range 320–236 nm.

3.6. Fluorescence Titration Measurements

Comparative studies on the native and 2 mM DTT-treated (reduced) *C. cajan* PI were performed by fluorescence emission spectroscopy. Fluorescence quenching data with two quenchers, acrylamide and potassium iodide, were analyzed according to (eq. 2) and (eq. 3). The Stern-Volmer plot and modified Stern-Volmer plot for native PI, reduced PI, and NATA with acrylamide and KI are depicted in Figures 5 and 6, respectively. K_{SV} and f_a values obtained for both samples are summarized in Table 4. Figure 5 shows the quenching results obtained with acrylamide. Acrylamide, which is a neutral and efficient quencher, quenches the fluorescence of tryptophan residues both exposed to the solvent and buried inside the protein. The Stern-Volmer for acrylamide is linear, i.e., all tryptophans in the PI are accessible to acrylamide. K_{SV} for reduced PI is greater (1.3×10^{-2} mM^{-1}) as compared to native PI (1.0×10^{-2} mM^{-1}) indicating that tryptophan residues in the reduced PI are more exposed to the solvent.

In order to understand more about the environment of tryptophan residues in *C. cajan* PI, we performed quenching studies with the anionic quencher, iodide ion (Fig. 6). According to many studies using ionic

Table 3. Effect of 2 mM DTT Treatment on the Inhibitory Activity of *C. cajan* PI

	Inhibitory Activity (%)
Untreated PI	85.00 ± 0.49
PI + DTT (30 min)	50.00 ± 5.66
PI + DTT (60 min)	18.00 ± 2.83

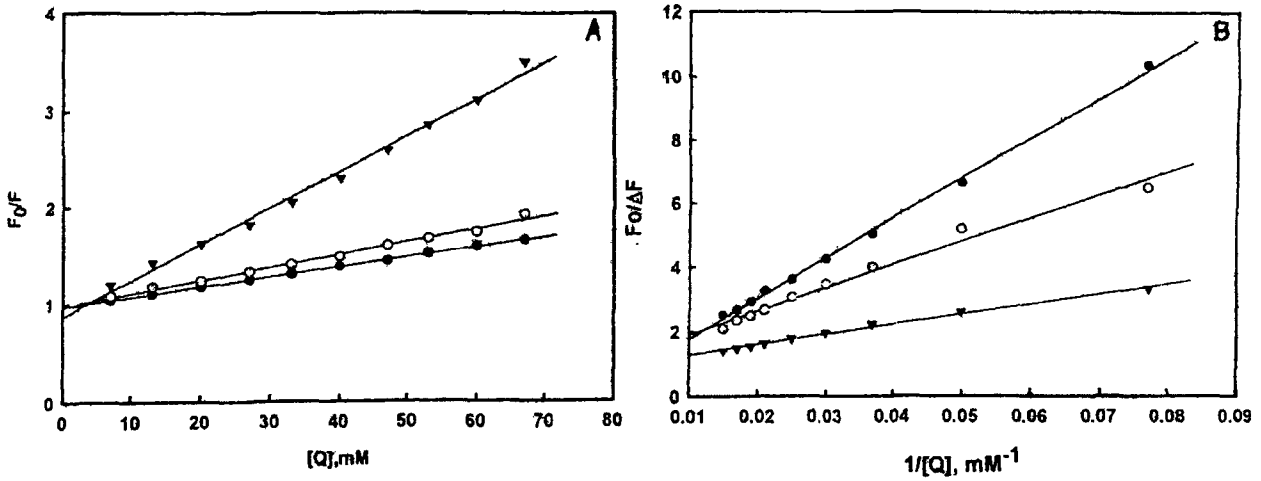

Fig. 5. Acrylamide quenching of tryptophan fluorescence for native *C. cajan* PI (—●—), reduced PI (—○—) and NATA (—▼—). (A) Stern-Volmer plot. (B) Modified Stern-Volmer plot.

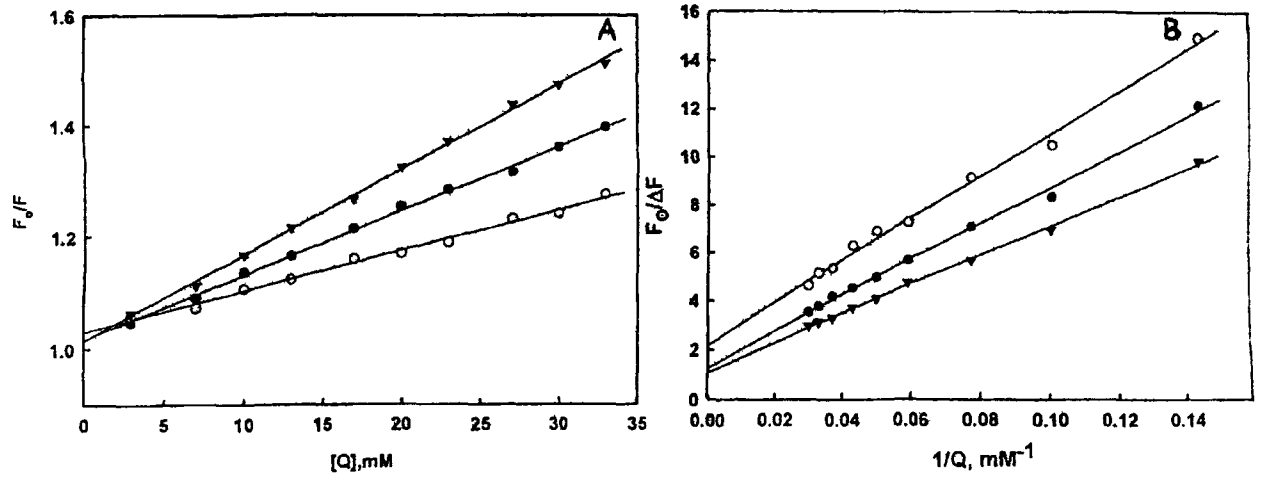

Fig. 6. KI quenching of tryptophan fluorescence for native *C. cajan* PI (—●—), reduced PI (—○—) and NATA (—▼—) (A) Stern-Volmer plot (B) modified Stern-Volmer plot.

Table 4. Fluorescence Parameters for Quenching of NATA, Native *C. cajan* PI and Reduced PI by Acrylamide and KI from Equations (2) and (3)

	K_{SV} (mM^{-1})		f_a	
Protein	Acrylamide	KI	Acrylamide	KI
NATA	0.037	0.015	0.988	0.926
PI (native)	0.010	0.012	0.507	0.787
PI (reduced)	0.013	0.007	1.178	0.455

quenchers like an iodide ion, it is well-known that the emission of exposed tryptophan residues are quenched selectively (Lakowicz, 1983; Somogyiet *et al.*, 1985). Iodide ion, being negatively charged and hydrated, is likely to quench only surface tryptophanyl residues, and its behaviour should also depend on the neighbouring charged groups (Lehrer and Learis, 1978). Contrary to acrylamide, KI quenching revealed a lesser degree of quenching in reduced PI as compared to native PI. This suggests that some tryptophans in the *C. cajan* PI are present in the vicinity of positively charged groups;

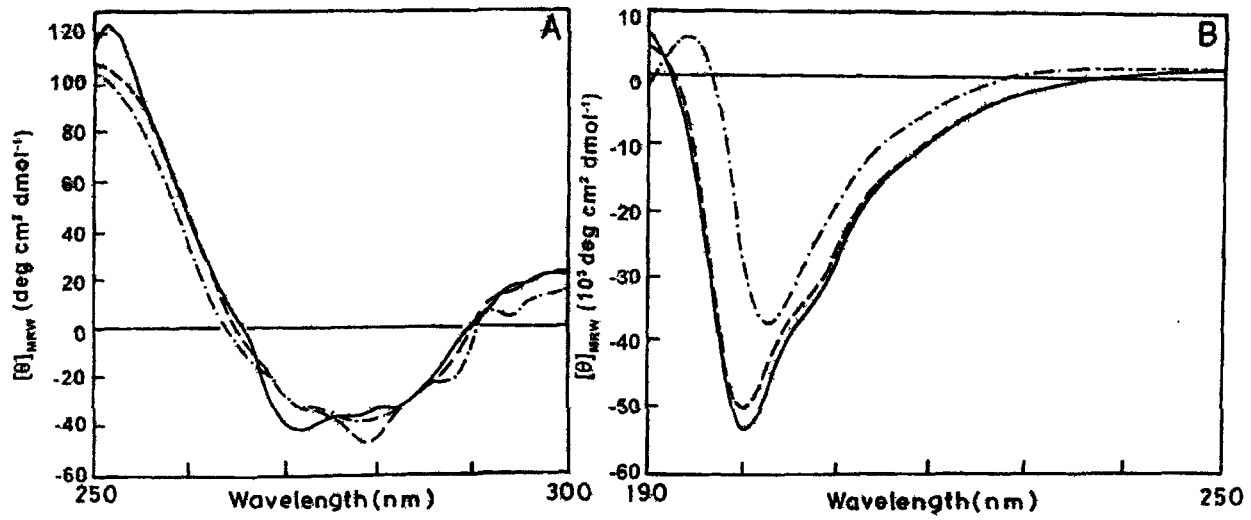

Fig. 7. Effect of pH on the conformation of *C. cajan* PI. Near-UV (A) and Far-UV(B) CD spectra of the PI at pH 7.0 (—), pH 2.0 (——) and pH 10.0 (—●—).

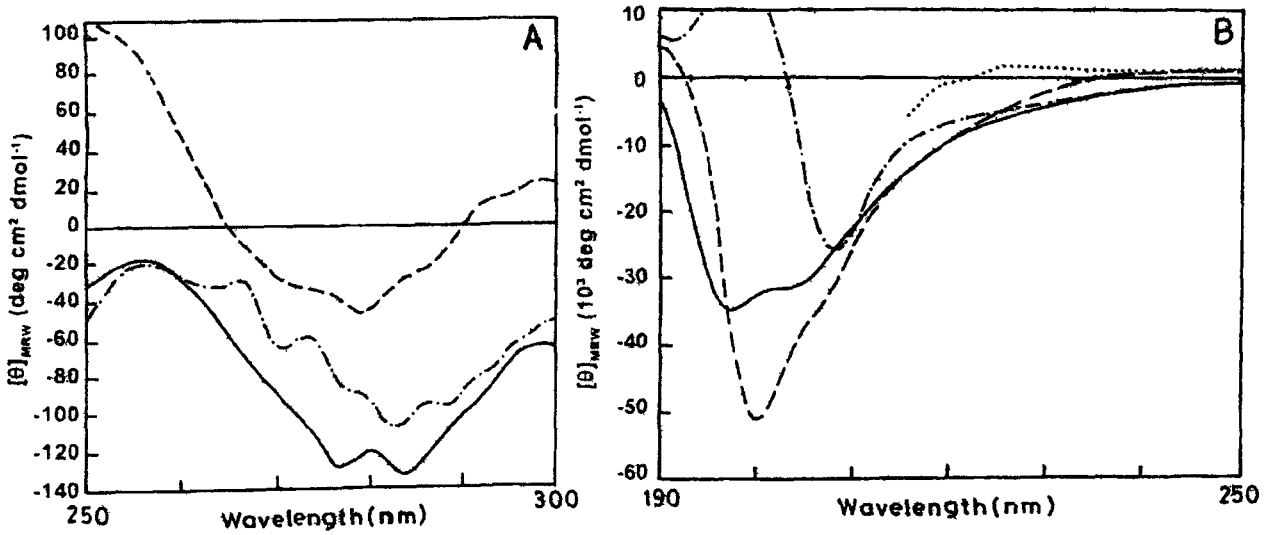

Fig. 8. Near-UV (A) and Far-UV (B) CD spectra of native *C. cajan* PI (——), PI + 1 mM DTT (——). PI + 10 mM DTT (—●—) and PI + 6 M Gdn. HCl (●●●●).

hence inaccessible to KI quenching. The percentage of the total fluorescence quenched by the ionic quencher calculated from the modified Stern-Volmer plot using Eq. 3 was found to be ~50%. This suggests that approximately half of the total number of tryptophans present in PI are exposed to solvent. NATA was used as a reference for free tryptophan and its quenching behaviour was similar in both cases. To rule out the effect of ionic strength we measured the fluorescence spectra for PI with varying [NaCl] in the range of 3.3–33 mM (same as that of KI). No significant change was observed on the intensity of the steady-state fluorescence spectra.

3.7. CD Spectroscopy

The near-UV CD spectrum shown in Figure 7 for *C. cajan* PI at pH 7.0 exhibits a minima at 279 nm which could be due to tyrosine residues as previously reported

for horsegram proteinase inhibitor by P. R. Ramasarma *et al.* (1994). A maximum at 250 nm is also observed which is attributed to disulfide linkages in the molecule (Venyaminov and Yang, 1996). In the far-UV CD spectrum (Fig. 7) of *C. cajan* PI at pH 7.0, a strong negative band centered at 200 nm and a positive band at 190 nm are observed. This is indicative of unordered structure with disulfide bonds as reported previously for Horse gram PI (Ramasarma *et al.*, 1994). The class of unordered proteins includes many oligopeptides, short polypeptides with disulfide bonds or prosthetic groups, and denatured proteins. These oligopeptides and polypeptides usually show a CD spectrum with a strong negative band near 200 nm, and some weak bands between 220 and 230 nm, which can have either positive or negative signs (Connelli *et al.*, 1985). Most of these inhibitory polypeptides show random coil secondary structure (Woody and Dunker, 1996).

A probe into the structural features of *C. cajan* PI at three different pH values, viz, 7.0, 2.0, and 10.0 yielded a largely stable conformation (Fig. 7). Near-UV CD spectra show minor conformational alterations at the pH values examined. In the far-UV CD spectrum observed at pH 2.0, *C. cajan* PI retains the observed CD bands at 200 nm and 190 nm; the depth of the negative band at 200 nm being slightly larger than that for pH 7.0. However, the spectrum is altered at alkaline pH. The positive band at 190 nm is lost and the 200 nm band is red-shifted. This suggests that the *C. cajan* PI is a relatively stable protein as its conformation is only slightly affected at low pH.

Table 5. Kinetic Parameters for the Interaction of *C cajan* PI with Trypsin and α-Chymotrypsin

Enzyme	$k_{ass}(M^{-1}\ s^{-1})$	K_i (M)
Trypsin	$(1.0 \pm 0.2) \times 10^6(3)^a$	$(2.8 \pm 0.1) \times 10^{-10}(3)$
α-Chymotrypsin	$(5.0 \pm 0.2) \times 10^3(3)$	$(4.0 \pm 0.3) \times 10^{-5}(3)$

[a] Numbers in parentheses denote the number of times the experiments were repeated

Figure 8 depicts the effect of DTT on the *C. cajan* PI. In the presence of 1 mM and 10 mM DTT, the PI clearly shows a loss in the 257 nm positive band which has been correlated to loss of disulfide linkages (Ramasarma *et al.*, 1994). The trough at 279 nm is abolished, and new minima appeared at 278 nm and 284 nm in the presence of 1 mM DTT indicating gross conformational alterations in the overall tertiary structure. Far-UV CD spectra also show pronounced conformational changes in the reduced PI samples. The positive

Table 6. The N-Terminal Sequence of *C cajan* PI Aligned with Other Known Kunitz-Type Trypsin Inhibitors

Inhibitor (Source)	Initial position	Sequence[a]	Reference/ NCBI_Tax ID
Erythrina variegata trypsin inhibitors			
ETIa	1	V **L** L D G	Kouzuma *et al*, 1992
ETIb	1	E **L** V D V	Kouzuma *et al*, 1992
Putative Kunitz-type tuber invertase inhibitor (Fragment) *Solanum tuberosum*	3	F **L** V **L** S	4113
Cajanus cajan proteinase inhibitor	1	G **L** V **L** D	
Soybean(*Glycine max*) Trypsin Inhibitor B (Kunitz)	1	D F **V** **L** D	3847
Kunitz inhibitor from *Peltophorum*	1	D F **V** **L** D	Joubert, 1981
Dimorphandra mollis trypsin inhibitor-II (DMTI-II)	1	**L** **V** Y D	Mello *et al*, 2002
Dimorphandra mollis trypsin inhibitor (DMTI)	2	Q **V** F D	Macedo *et al*, 2000
Kunitz-type serine protease inhibitor (BbKI) from *Bauhinia bauhinioides*	1	S V **V** V D	166014
α-chain of proteinase iso inhibitorDE5 from seeds of Carolina (*Adenanthera pavonina* L)	1	R E L **L** D	Richardson *et al*, 1986

[a] Symbols in bold type to highlight sequence homology positions aligned with respect to *C cajan* PI

band at 190 nm attributed to disulfides is lost in the presence of 1 mM DTT The strong negative band near 200 nm observed in native PI is greatly altered in the reduced preparations and disappears completely in the presence of 6 M Gdn HCl

The above discussed conformational alterations in the *C. cajan* PI as studied by CD spectroscopy undoubtedly suggest the role of disulfide linkages in the structure and function (as observed by decrease in inhibitory activity upon reduction) of the proteinase inhibitor

3.8. Kinetics of Binding to Serine Proteinases

The kinetics of association of *C cajan* PI to trypsin and α-chymotrypsin were analyzed by measurements of the loss of enzyme activity in the presence of specific chromogenic substrates All analyses showed a linear dependence of K_{obs}, on inhibitor concentration within the concentration range covered, the slopes of these plots giving k_{ass} (Table 5) The PI binds to trypsin with about 200-fold higher k_{ass} than to α-chymotrypsin The dissociation equilibrium constant, K_d, for the binding of *C cajan* PI to trypsin and α-chymotrypsin was determined as K_i by measurements of the decrease caused by the inhibitor of the equilibrium rate of cleavage of a chromogenic substrate by the respective proteinase (Table 5) As can be seen from the table, the affinity of *C cajan* PI for trypsin is much higher to that for α-chymotrypsin The main reason for the high affinity for trypsin is rapid association (k_{ass} $\sim 10^6$ M^{-1} s^{-1}), whereas the rate of binding to α-chymotrypsin is much slower

3.9. N-Terminal Sequencing

The N-terminal sequence of *C cajan* PI is given in Table 4. A comparison with other known inhibitors to check sequence homology revealed that it resembles Kunitz inhibitors especially Soybean trypsin inhibitor (Kunitz)

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Minireview

Protein proteinase inhibitor genes in combat against insects, pests, and pathogens: natural and engineered phytoprotection

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Abstract

The continual need to increase food production necessitates the development and application of novel biotechnologies to enable the provision of improved crop varieties in a timely and cost-effective way A milestone in this field was the introduction of *Bacillus thuringiensis* (*Bt*) entomotoxic proteins into plants Despite the success of this technology, there is need for development of alternative strategies of phytoprotection Biotechnology offers sustainable solutions to the problem of pests, pathogens, and plant parasitic nematodes in the form of other insecticidal protein genes A variety of genes, besides (*Bt*) toxins that are now available for genetic engineering for pest resistance are genes for vegetative insecticidal proteins, proteinase inhibitors, α-amylase inhibitors, and plant lectins This review presents a comprehensive summary of research efforts that focus on the potential use and advantages of using proteinase inhibitor genes to engineer insect- and pest-resistance Crop protection by means of PI genes is an important component of Integrated Pest Management programmes

Keywords Plant protein proteinase inhibitors Insect Nematode Virus, Genetic engineering

The current scenario

Losses of agricultural production due to pests and diseases have been estimated at 37% in Europe and worldwide In addition to crop damage caused by feeding insects, mites, and nematodes cause additional yield losses by carrying and infecting crops with disease causing pathogens Worldwide, over 200 plant diseases are known to be transmitted by insects, mites, and nematodes At present, crop protection relies predominantly on the use of environmentally toxic agrochemicals that are also deleterious to human health Instead of using chemical pesticides on a large scale, other alternatives need to be explored that are based on an interdisciplinary, system-oriented science and technology and are beneficial in terms of sustained agriculture Such sustainable systems will have decreased inputs of energy and chemicals, and will not generate harmful outputs such as pesticide residues [1] To achieve this objective, it is necessary to enhance the resistance of plants to pests and pathogens through Integrated Pest Management (IPM)[1] programmes that comprise a combination of control strategies including the judicious use of pesticides, crop rotation, field sanitation, and above all exploitation of inherently resistant plant varieties These farming practices fall under the various headings of Chemical Control, Biological Control, Cultural Control, Mechanical Control, and Genetic Control Genetic

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[1] *Abbreviations used Bt, Bacillus thuringiensis* VIPs, vegetative insecticidal proteins PIs proteinase inhibitors, AIs α-amylase inhibitors, IPM Integrated Pest Management, CpTI cowpea trypsin inhibitor STI, soybean trypsin inhibitor PCD programmed cell death TEV tobacco etch virus PVY potato virus Y MCPI metallocarboxypeptidase inhibitor GUS β-glucuronidase

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control or the use of transgenic crops [2], expressing foreign insecticidal genes, could make a significant contribution to sustainable agriculture and thus is an important component of IPM.

Current control relies predominantly on the use of transgenic *Bacillus thuringiensis* (*Bt*) crops. However, to combat against the broad variety of damaging insects and pests; plants need to strengthen their defense arsenal. The ubiquitous occurrence of proteinase inhibitors is a well-established fact. Proteinase inhibitors are natural, defense-related proteins often present in seeds and induced in certain plant tissues by herbivory or wounding [3,4]. The possible role of protease inhibitors (PIs) in plant protection was envisaged as early as 1947 when Mickel and Standish [5] observed that the larvae of certain insects were unable to develop normally on soybean products. PIs are present in multiple forms in numerous tissues of animals and plants as well as in microorganisms. PI genes are thus promising alternatives to the use of *Bt* insecticidal genes and in certain cases, may even complement it.

Methods in use: are they sustainable?

Various approaches used until this date include the use of:

(a) Chemical pesticides: a *curative* pest control strategy that is useful to immediately control certain pest outbreaks. However, excessive and indiscriminatory, large-scale use of pesticides leads to development of resistant plant varieties (*akin* to antibiotic resistance), besides having a harmful effect on the environment.
(b) Natural biocontrol factors such as pathogens, predators, or parasites; *antagonists* that follow various modes of action such as (i) competition for nutrients, (ii) competition for space, (iii) siderophore production, (iv) antibiosis, and (v) production of hydrolytic enzymes or other active substances. Examples of such biological control include, besides others, the use of non-pathogenic strain of *Ralstonia solanacearum* against its pathogenic isoform; the Vedalia beetle, *Rodollia cardinalis* against the cottony-cushion scale pest and the egg-parasitoid *Trichogramma evanescens* as a potential biological control agent against the Indian mealmoth *Plodia interpunctella* [6–8]. The complex modes of action exerted by many such biocontrol agents gives advantages over chemical pesticides in a possible hampering of the building up of resistance against them in the target pests [9].
(c) Various *preventive* pest control strategies employing crop rotation, intercropping, and cultivation of pest-resistant varieties of plants. It also involves the application of cultural control practices, such as the use of clean planting material, systematic trapping of adult insects (to control population build-up), and field sanitation (whereby residues that may form breeding grounds are removed).
(d) Transgenic insect-resistant crops expressing the insecticidal *Bt* toxins. Another insecticidal gene has been developed from the bacterium *Bacillus cereus*, known as the vegetative insecticidal protein ('*vip*') gene, that is effective against corn rootworms [6].

Bt toxin technology: pros and cons

At present, the main strategy to develop insect-resistant plants via genetic engineering is based on the use of *Bt* toxin genes. This has resulted in transgenic crops with a high resistance to specific insects. Current transgenic *Bt* crops expressing the Cry protein genes (crystal proteins or endotoxins) target key pests and also those resistant to conventional pesticides. Indeed, these proteins cumulate several advantages such as a high specificity, a short life in the environment, and a high and fast toxic activity [6,10,11]. Hence, it is no surprise that *Bt* makes up to 98% of all biopesticides and represents the quasi-exclusive source of pest-resistance genes for the development of transgenic plants. There are also significant environmental benefits such as absence of pesticide drift, absence of residual pesticide in soil, and absence of effects on non-target species. Since the toxin is expressed throughout its tissues, the transgenic plant effectively controls root pests, stem and fruit borers, and sucking insects unlike the conventional *Bt* sprays that could only protect the plant surface [6,12].

However, there are limitations to the use of transgenic *Bt* plants as well. Increased persistence of the *Bt* toxin within the plant throughout the growing season selects intensely for insect resistance [13]. Also, the range of insects which can be controlled by *Bt* toxins is relatively narrow. Moreover, *Bt* toxins have a fairly complex mode of action. Many of the protoxin classes need to be proteolytically activated after solubilization in the guts of susceptible insects. The activated toxins then bind to receptor(s) on insect midgut epithelial cells and eventually integrate into the membrane where they create ion channels. Disruption of the intracellular ionic homeostasis ensues leading eventually to cell death presumably by a colloid osmotic type mechanism [14,15]. No *Bt* toxin with adequate aphid toxicity has been described even as aphids continually cause significant crop losses. Hence, it is necessary and desirable to develop safer and more effective transgenic alternatives to reduce crop losses. One such alternative is to employ PI genes, especially in view of reports on proteinase-mediated insect resistance to *Bt* toxins [16].

PI genes: the advantage

Improved and extended genetic crop resistance is usually seen as a foremost possibility in the current scenario of preventing agricultural losses due to insects and diseases. Hence, another approach to slowing down insect growth is to use genes that encode for natural defensive compounds that are abundantly found in seeds, namely proteinase and α-amylase inhibitors. Subsequent to the preliminary observation of Mickel and Standish of the role of soybean products on crop protection, the trypsin inhibitors present in soybean were shown to be toxic to the larvae of flour beetle, *Tribolium confusum* [17]. Following these early studies, there have been many examples of protease inhibitors active against certain insect species, both in vitro assays against insect gut proteases [18,19] and in vivo artificial diet bioassays [20–22].

Moreover, since proteinase inhibitor genes are primary gene products, they are excellent candidates for engineering pest-resistance into plants [1]. Inhibitor genes of plant origin are particularly promising. This was first demonstrated by Hilder et al. [23] by transferring trypsin inhibitor gene from *Vigna unguiculata* to tobacco, which conferred resistance to wide range of insect pests including Lepidopterans, such as *Heliothis* and *Spodoptera*, Coleopterans, such as *Diabrotica* and *Anthonomnous*, and Orthoptera, such as Locusts. Following these early reports on the success of genetically engineered resistance in plants, several other PIs from diverse plant sources have been studied.

The availability of diverse genes from different plant sources is in itself an advantage as two or more genes can be transferred in combination (with different physiological targets) [24]. Proteinase inhibitors are also reportedly active against nematodes, viral, bacterial, and fungal pathogens; thus, they may serve to have a cumulative protective effect on plants. Further, there is no evidence that proteinase inhibitors have toxic or deleterious effects on mammals. These advantages make protease inhibitors an ideal choice to be used in developing transgenic crops resistant to insect pests.

PIs are effective against nematodes and other pathogens

Plant parasitic nematodes cause estimated annual losses to world agriculture of US $100 billion [25] and current control relies on the use of toxic, environmentally damaging nematicides such as methyl bromide and 1,3-dichloropropene (Telone II). Both are used as a preplant fumigant to protect the root system of young plants. The USA has committed itself to withdrawing the use of methyl bromide from agriculture as part of the Montreal Protocol. The withdrawal and restricted use of these chemical agents provides both an opportunity and a need for alternative control strategies.

Plant proteinase inhibitors are known to confer natural as well as engineered protection against nematode attack [26–29]. Several transgenic approaches to crop improvement have the potential to enhance crop resistance to nematodes [30]. Currently, an anti-feedant strategy, involving expression of proteinase inhibitors (PIs), offers the most advanced approach for nematode control. Cysteine PIs (cystatins) have been demonstrated as the most effective defense against the parasitic stage of *Rotylenchulus reniformis* [31] where the engineered rice cystatin (Oc-IDD86) reduced female fecundity and density when expressed in *Arabidopsis* under the control of CaMV35S promoter. Expression at similar levels of a serine PI from cowpea (CpTI) led to a smaller decrease in female density and no decrease in fecundity. Similarly, modified rice cystatin, Oc-I delta D86, when expressed as a transgene in *Arabidopsis thaliana*, has a profound effect on the size and fecundity of females for both *Heterodera schachtii* (beet-cyst nematode) and *Meloidogyne incognita* (root-knot nematode) [32]. The nematode gut cysteine proteinase activity was lost (anti-feedant defense) and no females of either species achieved the minimum size required for egg production. Another study [33] revealed that although the transgenic plants expressing protease inhibitors have enhanced levels of resistance to potato cyst-nematodes (PCN), *Globodera pallida* and *G. rostochiensis*, there was negligible effect on non-target herbivorous insect *Eupterya aurata*; suggesting minimal risk factors involved.

Oryzacystatins I and II are known to form complexes with cysteine proteinases from crude extracts of two-spotted spider mite, a plant pest [34], thus suggesting a possible role against mites as well. Transgenic crop plants expressing phytocystatins could also be used to suppress the growth rates of slug populations in the field as suggested by studies on a transgenic *Arabidopsis* leaf tissue expressing a modified oryzacystatin that exhibited resistance towards the field slug *Deroceras reticulatum* (Muller) [35].

PIs as novel antifungal agents

Proteinase inhibitors have also been implicated to play a role in the plant's natural defense towards fungal infections [36], further augmenting the potential uses of these insecticidal genes [37–39].

Plant cystatins are active against viruses

Constitutive expression of a rice cysteine proteinase inhibitor gene was found to induce resistance against two important potyviruses, tobacco etch virus (TEV)

and potato virus Y (PVY), in transgenic tobacco plants. Tobacco lines expressing the foreign gene at varying levels were examined for resistance against TEV and PVY infection and a clear, distinct correlation was observed between the oryzacystatin message, inhibition of papain (a cysteine proteinase), and resistance to TEV and PVY in all lines [40]. Certain soybean cultivars are also resistant to soybean mosaic virus owing to the presence of proteinase inhibitor genes [41].

PIs are also involved in regulation of PCD in plants

A novel role for proteinase inhibitor genes as modulators of programmed cell death (PCD) in plants has been proposed by Solomon et al. [42]. They have demonstrated that the ectopic expression of cystatin genes inhibited the induced cysteine protease activity and blocked PCD triggered indirectly by an avirulent strain of *Pseudomonas syringae* pv *glycinea* or directly by oxidative stress. In plants, programmed cell death has been implicated in xylogenesis [43,44], in some forms of senescence, and more importantly in the hypersensitive response to pathogens and environmental stresses [45–47]. This suggests that besides directly targeting pathogens, proteinase inhibitors also indirectly protect the plant from PCD as induced by such pathogen infestations.

Other evidence comes from studies on *AtCYS1*, a cystatin from *A. thaliana* which is constitutively expressed in roots and in developing siliques of *A. thaliana*; it is strongly induced in leaves by wounding, by challenge with avirulent pathogens, and by nitric oxide (NO). This overexpression of *AtCYS1* blocks cell death activated by either avirulent pathogens or by oxidative and nitrosative stress in both *A. thaliana* suspension cultured cells and in transgenic tobacco plants [48].

The inhibitor families

Laskowski and Kato have classified the proteinase inhibitors into several families based on extensive homology among its members, topological relationships between the disulfide bridges and the location of the reactive site. Plant serine proteinase inhibitors that obey the standard mechanism (as discussed later) are grouped into soybean (Kunitz), Bowman–Birk, potato I and II, and squash families [49]. Several other inhibitor families, such as barley, ragi 1 and 2, and thaumatin, have also been suggested [3,50] (refer to Table 1).

Inhibitors with class-specific reactive sites

The inhibitors discovered so far have been found to be specific for each of the four mechanistic classes of proteolytic enzymes, and based on the active amino acid in their "reaction center" [52] are classified as serine, cysteine, aspartic, and metallo-protease inhibitors. The activity of PIs is due to their capacity to form stable complexes with target proteases, blocking, altering or preventing access to the enzyme active site.

Table 1
The inhibitor families of plant origin[a]

	Name	Reference
I	Soybean trypsin inhibitor (Kunitz) family	[51]
II	Soybean trypsin inhibitor (Bowman–Birk) family	[52,53]
III	Potato I inhibitor family	[54]
IV	Potato II inhibitor family	[55]
V	Squash family	[56]
VI	Other families	
	Barley family	
	Cereal superfamily	
	Thaumatin-like inhibitors	
	Ragi A1 inhibitors	

[a] Adapted from Laskowski and Kato [49] with slight modification

Serine proteinase inhibitors

Serine proteinase inhibitors are universal throughout the plant kingdom and have been described in many plant species. Therefore, the number of known and partially characterized inhibitors of serine proteinases is enormous. Serine proteinase inhibitors have been reported from a variety of plant sources [57–68] and are the most-studied class of proteinase inhibitors. Serine proteinase inhibitors are widespread in the plant kingdom, their physiological roles including the regulation of endogenous proteinases during seed dormancy, the reserve protein mobilization, and the protection against the proteolytic enzymes of parasites and insects. Moreover, they may also act as storage or reserve proteins.

The two best-characterized families of plant serine proteinase inhibitors are the Kunitz-type and Bowman–Birk inhibitors. Kunitz-type inhibitors have a molecular mass of 18–22 kDa, one or two polypeptide chains, a low cystine content (usually with four Cys residues in two disulfide bridges), and one reactive site. In contrast, Bowman–Birk type inhibitors have a lower molecular mass (8–10 kDa), a high cystine content, and two reactive sites [69].

Cysteine proteinase inhibitors

Plant cystatins or phytocystatins are the second most-studied class of inhibitors and have been identified and characterized from several plants, viz., cowpea [70], potato [71], cabbage [72], ragweed [73], carrot [74], papaya [75], apple fruit [76], avocado [77], chestnut [78], Job's tears [79], etc. Cystatins have also been isolated from

seeds of a wide range of crop plants. These crop plants include those of sunflower [80], rice [81], wheat [82], maize [83], soybean [84], sugarcane [36], etc. Of the PI-expressing transgenic plants produced until this date, those expressing cysteine proteinase inhibitors have shown the most promising results, probably because most phytophagous insects employ these proteinases in their digestive mechanism.

Aspartic proteinase inhibitors

Aspartic proteinase inhibitors are a relatively less-studied class partly due to their rarity of occurrence. Potato tubers possess an aspartic proteinase (cathepsin D) inhibitor [85] that shares considerable amino acid sequence identity with the trypsin inhibitor SBTI from soybeans. It is the only well-characterized cathepsin D inhibitor of protein character.

Metallo-proteinase inhibitors

The metallo-proteinase inhibitors in plants are represented by the metallo-carboxypeptidase inhibitor family in tomato [86] and potato plants [87,88].

PIs inhibit enzymes mostly by mimicking substrates

Extensive research on proteinase inhibitors has provided a basic understanding of the mechanism of action that applies to most serine proteinase inhibitor families and probably to the cysteine and aspartyl proteinase inhibitor families as well. All serine inhibitor families from plants are competitive inhibitors and all of them inhibit proteinases with a similar standard mechanism as proposed by Laskowski and Kato [49]. Inhibition occurs as a consequence of binding of the active-site substrate-binding region of a proteinase to the corresponding substrate-like region (reactive site) on the surface of the inhibitor.

Therefore, inhibitors behave as highly specific, limited proteolysis substrates for their target enzymes. On the surface of each inhibitor molecule lies at least one (more in multiheaded inhibitors) peptide bond called the reactive site [89], which specifically interacts with the active site of the cognate enzyme. The value of k_{cat}/K_m for the hydrolysis of this peptide bond by the cognate enzyme at neutral pH is very high, 10^4–10^6 M^{-1} s^{-1} [90], compared to a typical value for normal substrates of about 10^3 M^{-1} s^{-1}. However for inhibitors, the values of k_{cat} and K_m are both many orders of magnitude lower than the values for normal substrates. Therefore, their hydrolysis is extremely slow, and the system behaves as if it were a simple equilibrium between the enzyme and free inhibitor on the one hand and the complex on the other.

The reactive site peptide bond after hydrolysis (i.e., in the modified inhibitor) acquires a newly formed carboxyl terminal residue designated as P_1 and a newly formed amino terminal residue designated as P'_1. The reactive site residue P_1 generally corresponds to the specificity of the cognate enzyme. Thus, inhibitors with P_1 Lys and Arg tend to inhibit trypsin and trypsin-like enzymes, those with P_1 Tyr, Phe, Trp (artificial only), Leu, and Met inhibit chymotrypsin and chymotrypsin-like enzymes, and those with P_1 Ala and Ser inhibit elastase-like enzymes.

On the basis of elegant studies on various crystal structures of enzyme–inhibitor complexes Bode and Huber [91], showed that inhibitors bind to their cognate enzymes in either a "substrate canonical form" or a "product canonical form."

Induction of PI synthesis and its regulation in plants

Plants produce protease inhibitors in response to feeding or wounding that are usually active against the endoproteases [3]. The protease inhibitors are also developmentally regulated as has been observed in case of cabbage and sweet potato [92–94]. Highest levels are found in very young leaves while older leaves have the lowest levels, also higher in the cabbage head.

Earlier research indicated that wound-induced accumulation of proteinase inhibitors in tomato was mediated by a proteinase inhibitor initiation factor (PIIF) that switched on a cascade of events leading to the synthesis of the inhibitor proteins [95,96] that were primarily cytosolic [97,98]. As reported by Lawrence and Koundal [99], in their review, current evidence suggests that the production of the inhibitors occurs via the octadecanoid (OD) pathway, which catalyzes the breakdown of linolenic acid and the formation of jasmonic acid (JA) to induce proteinase inhibitor gene expression. There are four systemic signals responsible for the translocation of the wound response (through the xylem or phloem), which includes systemin, abscisic acid (ABA), hydraulic signals (variation potentials), and electrical signals [100–102]. Evidence of systemin playing a key role in systemic signaling came from the pioneering work of C.A. Ryan. By showing that tomato plants expressing an antisense prosystemin gene become deficient in long-distance signaling and are more susceptible to insect attacks than wild-type plants [103], an important role of systemin in defense signalling pathways was clearly established. Immunolocalization techniques revealed that prosystemin, the precursor of systemin found in several plants [104], is localized in parenchyma cells of vascular bundles [105], this localization in the vicinity of the sieve tubes of the phloem may facilitate transport of systemin and

oxylipins it induces in response to wounding to distal cells. The activation of defensive genes by systemins involves a complex intracellular signaling pathway akin to the inflammatory response in animals. Systemin interacts with a 160 kDa cell surface receptor (SR160) that leads to the activation of a mitogen-activated protein kinase (MAPK) [106–108], the rapid alkalinization of the extracellular medium [109], the activation of phospholipase [110,111] and the release of linolenic acid that is converted into oxylipins such as phytodienoic acid and jasmonic acid that are powerful signals for defense genes [112,113]. Transgenic potato plants overexpressing the prosystemin gene were found to regulate the synthesis and accumulation of proteinase inhibitors in leaves [114]. Paralogous to the induction and synthesis of tomato inhibitor-II in tomato leaves in response to wounding, tobacco leaves synthesize a tobacco trypsin inhibitor (TTI) in response to wounding, suggesting a similarity between the wound signaling systems of the two plants. The search for a systemin-like signaling molecule by Ryan and Pearce [115] yielded tobacco hydroxyproline-rich proteins (TobHypSys) that were identified as members of a functionally related systemin family.

Mechanism of action in insect guts

There are differing views on the mechanism of development of resistance. Reese [116] had proposed the simple mechanism that growth rates were reduced due to reduced rates of proteolysis that was later dismissed when Broadway and Duffey [117] suggested that a feedback mechanism was leading to the hyperproduction of proteinases to compensate for the loss of activity, which in turn led to the depletion of essential amino acids and finally resulted in retarted growth rates. It was found that rats and chicks fed on low soya protein or STI extract developed hyperactive pancreas and their intestines contained greater amounts of pancreatic enzymes including trypsin even though a large percentage was inhibited. So depression of growth was not due to blocking of proteolysis, but to hyperactive pancreas. Nevertheless, the primary site of action of these inhibitors is the digestive system of insect larvae.

Since trypsin is involved in developmental processes such as molting and synthesis of neuropeptides, trypsin inhibitors can disrupt these processes thereby retarding growth and development of the larvae [17,118,119]. In a study conducted by Marchetti et al. [120], it was observed that larvae fed on transgenic plants expressing a Kunitz inhibitor, gradually lost their turgor and became shrunken; hence it appears that food avoidance also has a dramatic effect on the water balance of the feeding larvae.

Nutritional significance

Since inhibitor blocks intestinal protease, at first consideration one is led to think that PI would cause malabsorption of amino acids and lack of growth in animals. However, studies on a range of experimental animals show that this is not the case. Although STI inhibits almost all trypsins in vitro, it does not cause poor growth in all animals. STI blocks trypsin leading to pancreas feedback mechanism; pancreas goes into hyper-production of pancreatic enzymes causing rapid depletion/loss of essential nutrients. However, it is only a serious problem in animals with a simple feedback/control of pancreas secretion. Humans and cattle have more sophisticated systems involving several other proteases; thereby eliminating doubts of growth limiting problems in mammals [121]. There is no evidence that PIs have toxic or deleterious effects on mammals. Rather, they are known to improve the nutritional quality [122]. Also, since it is well known that legume proteins are deficient in the sulfur containing amino acids [123], Biermann et al. [124] attempted to increase the sulfur content by adding Met or Sulfate that in turn led to increased mRNA levels of PIs; thus, suggesting a positive role of PIs in nutrition. Many of these protease inhibitors are rich in cysteine and lysine, contributing to better and enhanced nutritional quality. Large amounts of inhibitors are normally present in many raw foods of plant origin, but their anti-nutritional effect is drastically reduced with simple technological treatments such as cooking. The oral administration of small quantities of certain inhibitors (e.g., soybean Bowman–Birrk) has a protective role against carcinogenesis in the esophagus, duodenum, and colon [125,126].

Insect gut proteinases: the target enzymes

For an efficient management of pest control through proteinase inhibitor transgenes, it is imperative to know the type of enzymes present in the guts of insects and pests. The two major proteinase classes in the digestive systems of phytophagus insects are the serine and cysteine proteinases. Murdock et al. [127] have made an elaborate study of the midgut enzymes of various pests belonging to Coleoptera (refer to Table 2). Many Lepidopterans have serine proteinases as the major digestive enzymes, whereas Coleopteran species have a wider range of dominant gut proteinases [1,128–132]. Cysteine proteinases account for most of the proteolytic activity in the gut of *Callosobruchus maculates* (F.) (Coleoptera; Bruchidae) [133–135], and a cathepsin D like proteinase has been identified in the posterior midgut of Hemipteran insects [136]. The larval midgut of Western corn rootworm (*Diabrotica vergifera* vergifera) also possesses cathepsin L like digestive cysteine proteinases [137].

Table 2
Insect gut proteases: mechanistic class/specificity

Insect		Major gut enzymes
Coleoptera		
Red fluor beetle	*Tribolium castaneum* (Herbst)	Serine/cysteine [127]
Mexican bean beetle	*Epilachna varivestis* (Mulsant)	Cysteine [127]
Cowpea weevil	*Callos obruchus maculatus* (L)	Serine/cysteine [133,135]
	Callosobruchus chinensis	Cysteine [143]
Cotton Boll weevil	*Anthonomus grandis* (Boheman)	Serine/cysteine [144]
Colorado potato beetle	*Leptinotarsa decemlineata* (Say)	Cysteine [127]
Sap beetle	*Glischrochilus quadrisignatus* (Say)	Cysteine [127]
Three-lined potato beetle	*Lema trilineata* (Olivier)	Cysteine [127]
Milkweed beetle	*Tetraopes tetraophthalamus* (Forster)	Serine
	Diabrotica virgifera	Cysteine [137]
Cigarette beetle	*Lasioderma serricorne* (Fabricius)	Serine [145]
	Acanthoscelides obtectus (Say)	[138]
Banana weevil	*Cosmopolites sordidus*	Cysteine [127]
Lepidoptera		
Tobacco hornworm	*Manduca sexta*	Serine
	Mamestra configurata	Serine [146]
	Anagasta kuehniella	Serine [66]
European corn borer	*Ostrinia nubialis* (Hubner)	Serine [119,147]
Angoumois grain moth	*Sitotroga cerealella* (Oliver)	[139]
Stalk corn borer	*Sesamia nonagrioides*	Serine [142]
	Spodoptera littoralis	Serine [140,143]
	Spodoptera exigua (Hubner)	[131]
	Satrotes subfasciatos	[148]
	Heliothes virescens	Serine [129]
Diptera		
Hessian fly	*Mayetiola destructor* (Say)	Serine [118]
Stable fly	*Stomoxys calcitrana* (L)	[149]
Hemiptera		
Plant sucking bugs	*Anasa tristis* (De Geea)	Cysteine [136]
	Coriscus pilosulus (Herrich-Schaeffer)	Cysteine [136]
Rice brown planthopper	*Nilaparvata lugens*	Cysteine [150]
	Reportus clavatus	Cysteine [151]

Most of the gut proteinases of *Acanthoscelides obtectus*, bruchids are cysteine and aspartic proteinases [138]. Characterization of the midgut enzymes of *Sitotroga cerealella* (Oliver), the Angoumois grain moth, and *Spodoptera littoralis* (Boisduval), a noctuid Lepidopteran revealed that the major digestive proteases were predominantly serine proteinases [139,140]. Thus, the digestive proteinases in insects are diverse [141,142]. Cysteine-type proteases were shown to form the major endoproteolytic system in the aphid digestive tract, and therefore cystatins are found to be more effective against these pests.

PI genes in combat against insects and pests: the success story

Following several mechanistic studies on transgenic proteinase inhibitors, it was found that serine PIs are effective against Lepidoptera [152,153] whereas cysteine PIs are effective against some Coleoptera [154,155]. Aphids constitute an important group of insect pests, even though a limited number of genes encoding aphid-efficient toxins are available. Since aphids feed on a low-protein diet (phloem sap), and do not rely on protein digestion for their nitrogen nutrition, the general absence of activity of PIs in early screening tests was not a surprise. However, several results obtained in vitro highlighted the interest of some members of this wide peptide class [151,156–158]. Initially, one PI member of the plant cysteine protease inhibitors, oryzacystatin (gene OC I), showed a low but significant growth inhibition activity on many aphid species tested in vitro. Furthermore, transgenic oilseed rape plants expressing OC I in their phloem sap affected similarly the larval growth of *Myzus persicae*, and induced significant reductions in aphid fecundity [159,160]. Finally, tissue and enzyme targets of OC I were successfully identified by immunolocalization and enzymatic assays on OC I-fed aphids and cysteine-type proteases were shown to form the major endoproteolytic system in the aphid digestive tract. Second, a pea seed Bowman–Birk trypsin/chymotrypsin inhibitor was shown to induce significant in vitro toxicity towards the pea aphid

Table 3
Transgenic plants carrying protein proteinase inhibitor genes in defense against insects and pests

Source gene	Transformed plant	Defense against	Reference
Proteinase inhibitor genes			
Cowpea trypsin inhibitor (CpTI)	Tobacco	*Heliothis virescens* larvae	[162]
	Tobacco	*Spodoptera litura*	[163]
	Cotton	*Helicoverpa armigera*	[164]
	Rice	Rice stem borers *Chilo suppressalis* and *Sesamia inferens*	[165]
	Potato	Tomato moth *Lacanobia oleracae*	[166]
	Strawberry	Vine weevil *Otiorynchus sulcatus* F	[167]
	Cabbage	*P rapae*	[168]
	Pigeonpea	*Helicoverpa armigera*	[169]
CpTi and snowdrop lectin	Sweet potato	*Cylas formicarius*	[170]
	'Jewel' sweet potato	West Indian sweet potato Weevil *Euscepes postfasciatus*	[171]
Soybean serine-proteinase inhibitor (C-II)	Potato/tobacco	Coleoptera/Lepidoptera	[121]
Soybean (Kunitz) trypsin inhibitor	Tobacco	*Spodoptera litura* larvae	[172]
	Rice	Brown planthopper *Nilaparvata lugens* Stal	[150]
Soybean Kunitz trypsin inhibitor (SKTI-4)	Sweet potato	*Cylas* spp	[173]
Soybean Kunitz, C-II and PI-IV inhibitor	Potato/tobacco	*Spodoptera littoralis*	[120]
Trypsin inhibitor from *Vigna unguiculata*	Tobacco	*Heliothis* *Spodoptera* Diabrotica Anthonomnous Locusts	[23]
Tomato proteinase inhibitors I and II	Tobacco	*Manduca sexta* larvae	[174]
Tomato proteinase inhibitor I	Nightshade	—	[175]
	Tobacco	—	[175]
	Alfalfa	—	[175]
Proteinase inhibitor SaPIN2a	Lettuce	—	[176]
Potato inhibitor II gene	Tobacco	*Chrysodeixus eriosoma*	[152]
	Rice	Pink stem borer *Sesamia inferens*	[177]
	Sugarcane	Sugarcane grubs *Antitrogus consanguineus*	[178]
Sweet potato (*Ipomea batatas*) trypsin inhibitor	Tobacco	*Spodoptera litura*	[153]
Trypsin inhibitor from barley (CMe)	Indica and japonica rice	Rice weevil *Sitophilus oryzae*	[179]
	Barley	*Agrotis ipsilon*	[180]
	Tobacco	Lepidoptera	[180]
	Wheat	*Spodoptera lituralis*	[181]
Mustard trypsin inhibitor-2	—	*Spodoptera littoralis* larvae	[182]
Mustard trypsin inhibitor (MTI-2)	Tobacco	*Plutella xylostella* (L)	[183]
	Arabidopsis	*Mamestra brassicae* (L)	[183]
	Oilseed rape	*Spodoptera littoralis* (Boisduval)	[183]
Oryzacystatin I	Poplar	*Chrysomela tremulae* (Coleoptera Chrysomelidae)	[155]
	Potato	Peach potato aphid *Myzus persicae*	[184]
	Potato	Colorado potato beetle larvae *Leptinotarsa decemlineata*	[154]
	Oilseed rape	Cabbage seed weevil (Coleoptera Curculionidae)	[185]
	Oilseed rape	*Myzus persicae*	[161]
Corn cystatin	Rice	Maize grain weevil *Sitophilus zeamais*	[186]
Nicotiana alata protease inhibitor	Tobacco	*Helicoverpa punctigera*	[187]
	Pea	*Plutella xylostella*	[188]
Arabidopsis thaliana cysteine proteinase inhibitor	White poplar (*Populus alba* L)	Chrysomelid beetle *Chrysomela populi*	[189]
PI genes against other pathogens (virus/nematode/etc)			
Modified oryzacystatin	*Arabidopsis thaliana* leaf tissue	Field Slug *Derocerus reticulatum*	[35]
	Arabidopsis	Beet-cyst nematode *Heterodera schachtii*	[32]
		Root-knot nematode *Meloidogyne incognita*	[32]
Cysteine PI	Tobacco	Potyviruses	[40]
Oryzacystatin	Transgenic hairy roots	*Globodera pallida*	[31]

Table 3 (*continued*)

Source gene	Transformed plant	Defense against	Reference
Oryzacystatin I and II	Alfalfa	Root-lesion nematode	[190]
Oryzacystatin I delta D86	Rice	Nematode resistance	
		Meloidogyne incognita	[191]
		Rotylenchulus reniformis	[31]
PIs from other organisms expressed in plants			
Manduca sexta proteinase inhibitor	Cotton	—	[192]
	Tobacco	*Bemesia tabaci*	[193]
Bovine spleen trypsin inhibitor	Tobacco	*Helicoverpa armigera* larvae	[194]
Where PIs have proved ineffective			
Barley trypsin inhibitor (BTI-CMe)	Tobacco	*Spodoptera exigua* larvae (Lepidoptera. Noctuida)	[195]
Trypsin PI	Tobacco/Arabidopsis	*Spodoptera littoralis* larvae	[196]
Soybean trypsin inhibitor	Tobacco	*Helicoverpa armigera*	[197,198]
Oryzacystatin	Oilseed rape	Beetle larvae *Ceutorynchus assimilis*	[199]
α-Amylase inhibitors in plant defense			
Bean α-amylase inhibitor I	Azuki bean	Bruchid resistance	[200]
	Pea	Pea weevil *Bruchus piorum*	[201]
	Pea	Bruchid beetle	[202]

Acyrthosiphon pisum [161]. Transgenic crops, including rice, expressing foreign PIs, show reduced predation by their natural pests. Table 3 shows some transgenic plants that have been genetically transformed to include protein proteinase inhibitor genes and their efficacies against the natural pests, nematodes, and other pathogens of the host plants.

Genetic Engineering: some aspects

Methods of gene transfer

Plant genetic transformation is a potential tool to understand genome regulation and allows for manipulation of genes that could otherwise not be done by classical breeding. Plant genetic engineering has come of age; it is now possible to address relationships between proteinase inhibitors and the growth, development, and survival of insect species in transgenic plants, where effects of single gene changes can be observed. Aspects of inhibitor specificity, insect proteinase specificity, physiological effects of proteinase inhibitor complexes or feedback mechanisms in insect digestion, and the roles of combined or synergistic effects of defensive genes can be assessed [152]. Further, transformation of plant genomes with PI-encoding cDNA clones appears attractive not only for the control of plant pests and pathogens, but also as a means to produce PIs, useful in alternative systems and the use of plants as factories for the production of heterologous proteins [203].

The general methods of gene transfer commonly employed are:

(1) By using the *Agrobacterium tumefaciens* mediated gene transfer method [150,204–206].
(2) PEG-mediated direct gene transfer to protoplasts of several cultivars [207–209].
(3) Electroporation-mediated DNA delivery [210].
(4) Microprojectile-mediated gene transfer (biolistic method) [211,212].

Choice of promoters and marker genes

Control and regulation of transgene expression are required for efficient gene transfer in transgenic plants. This regulation can occur during different steps of gene expression but more particularly during transcription and promoters ensure this control. Promoters with spatial and temporal expression patterns need to be studied to investigate and ultimately manipulate responses to biotic and abiotic stresses [213]. Selectable "marker genes" and efficient promoters are also introduced alongside the gene to identify transformed plant cells from untransformed ones and to ensure efficient transcription of the gene [214,215]. Conventionally "antibiotic-resistance" marker genes have been used; but due to rising concerns with their use, they are now being replaced with herbicide-tolerance genes (e.g., chlorsulfuron) that can eventually be bred away.

Characterization of inducible and/or tissue-specific promoters is important to achieve a better control of the expression of pest resistance genes. Examples of such wound inducible and/or fungal pathogen responsive promoters were those derived from rice *ltp* gene (lipo transfer protein), *hrgp* (hydroxyproline-rich glycopro-

tein), and *mpi* (maize protease inhibitor) genes from maize [216–218]. Vascular tissue-specific promoters from MSV (maize streak virus) and *A. thaliana* whereas fruit- and seed-specific promoters from wheat and *A. thaliana* [219] can be potentially used with transgenic plants for tissue-specific expression of genes.

The promoter regions of metallocarboxypeptidase inhibitor (MCPI) gene in tuberizing and non-tuberizing *Solanum* species are able to confer tuber- and berry-specific expression for the β-glucuronidase reporter gene in potato [220]. Thus, the MCPI genes are also potentially suitable for biotechnological application in potato to provide specific transgene expression in tuber and berry. Other promoter elements have also been identified that are involved in environmental and developmental control of potato proteinase II expression [221].

A number of reporter genes have been used as convenient markers to visualize gene expression and protein localization in a wide spectrum of prokaryotes and eukaryotes. The *Escherichia coli gus* reporter gene (also referred to as *uidA*) which encodes β-glucuronidase (GUS) has been routinely and extensively used in plants as an invaluable tool to follow gene transfer [222]. Recently, the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* was reported to function as a sensitive reporter of in vivo gene expression [223,224]. The fluorescent pattern of *gfp* is a useful, new tool for monitoring in vivo transgene expression in actinorhizal plants of the *Casuarinaceae* family [225] (refer to Table 4).

Insect resistance: getting prepared

To overcome protease inhibitor resistance of insects; it is now necessary to further develop protease inhibitors that are broadly active against all proteases that insects use for digestion. The objective is achieved by:

(1) **selecting** second generation protease inhibitors (novel PIs) from novel sources (unrelated organisms, synthetic libraries or insect haemolymph) with maximum effectiveness against the selected target pests; introducing novel PIs into important crop plants; and demonstrating, in the field or greenhouse, that a substantial reduction in the application of insecticides can be achieved.
(2) Use of bifunctional α-amylase/trypsin inhibitors is of particular practical interest since transgenic plants expressing a molecule which inhibited both amylases and proteases of pests would be highly protected. The presence of both the activities would also help to minimize the likelihood of the appearance of resistant pest strains.
(3) Another approach may be to express two or more PIs as a fusion protein. Expression of a cystatin and a serine PI in this way has been found to be successful against certain nematode pathogens [24].
(4) Studies on the insecticidal activity of transgenic tobacco plants expressing both *Bt* insecticidal protein and cowpea trypin inhibitor (CpTI) genes against cotton bollworm (*Helicoverpa armigera*) revealed that it was more effective compared to transgenic tobacco expressing *Bt* insecticidal protein gene alone. Besides the enhancement of insecticidal efficacy, insect adaptation to transgenic *Bt* crops is also delayed [227].

Some doubts and concerns

The level of pest control produced by a plant trait can be influenced by any genetic or environmental factors that affect primary or secondary metabolism of the crop. Thus, genetic background of a variety, the weather, soil fertility, moisture stress or insect injury, to name a few, can all influence effectiveness of plant produced pesticides, or any other gene product of the crop plant [6]. Besides this, transgenic expression has other hurdles to cross:

(i) Expression may be developmentally regulated.
(ii) Gene silencing: transformed genes become inoperative.
(iii) Multiple gene copies may be incorporated.
(iv) Gene may be incorporated at different sites.
(v) Environmental conditions such as heat, water stress, etc.

Table 4
Some promoters used with insect-resistance (enzyme inhibitor) genes[a]

Promoter sequence	Insecticidal protein	Expression site	Plant
Cauliflower mosaic virus 35S promoter (CaMV 35S)	Most proteinase inhibitor genes	Most plant tissues	Refer Table 3
Rice actin-1 promoter (Act-1)	CpTi	All plant organs	Rice [165], monocots
Potato proteinase inhibitor-II promoter (Pot PT-IIK)	Pot PT-II, ipt	Wound-inducible	Rice [177], tobacco [226]; tomato [226]
Bean phytohaemagglutinin promoter (PHA-L, dlec2)	α-AI-Pv	Seed	Pea [201,202]; azuki bean [200]
Patatin B33 promoter			
Potato Lhca 3.St1 promoter			
Chrysanthemum RbcS1 Rubisco small subunit promoter			

[a] Adapted from [216] with some modification

Other environmental and public health concerns

There are yet other environmental and public health concerns, voiced over the use of transgenic plants in general. These are:

(i) Cross-pollination of GM crop varieties with conventional varieties.
(ii) Germination of volunteer GM seeds (seeds dropped, blown or inadvertently planted).
(iii) Potential gene flow to other organisms.
(iv) Destruction of agricultural diversity.
(v) Allerginicity.
(vi) Antibiotic resistance.
(vii) Gastrointestinal problems.

Of these, the last two have been addressed to some extent by the use of herbicide-tolerant genes and studies on the nutritional aspects of proteinase inhibitors on mammals.

Conclusions

With the development of disease-, insect-, and drought-resistant crops, genetic engineering has addressed at least some of the environmental problems associated with conventional agriculture. With the increasing demand for food for the burgeoning human population world-wide and decreasing cultivable land, it appears that plant genetic engineering has to be adopted for maximum benefits in a minimum input. Losses due to pests have to be minimized and development of transgenic, insect- and pest-resistant crop varieties through proteinase inhibitor genes will make a promising contribution towards maximizing yields. However, as aptly pointed out by Lyson [7], agricultural biotechnologies are anchored to a scientific paradigm rooted in experimental biology, whereas sustainable agriculture rests on a biological paradigm that is best described as ecological. Nevertheless, there are still a lot of promising possibilities of pest control through insecticidal genes that need to be explored and prudently tapped for their implementation in IPM programmes.

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